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Herbert Dinsmore Madill

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A STUDY OF BRAIN ACETYLCHOLINE LEVELS AND THEIR RELATIONSHIP
TO THE TREMOROGENIC EFFECT OF NICOTINE-N-OXIDE IN MICE

by

Herbert Dinsmore Madill

Department of Pharmacology

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Canada.

February 1970

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ABSTRACT

In mice previously treated with sub-tremorogenic doses of the cholinesterase inhibitors DFP (diisopropyl phosphonofluoridate) and soman (pinacolyl methylphosphonofluoridate) there was a potentiation of the tremorogenic effect of intracerebrally injected oxidized nicotine and nicotine-N-oxide. This same effect did not occur when nicotine was similarly injected after treatment with these anticholinesterases. Atropine was shown to be ineffective in blocking the tremor produced by nicotine-N-oxide yet was able to remove the potentiating effect of DFP upon the tremorogenic effects of this compound and that of oxidized nicotine. Hemicholinium-3 did not affect the tremorogenic action of nicotine-N-oxide yet was able to greatly reduce the potentiating effect produced by DFP pretreatment. Mecamylamine was demonstrated to be an effective antagonist of the tremorogenic effects of i.c. injected nicotine, oxidized nicotine and nicotine-N-oxide but not acetylcholine. The blocking effects of mecamylamine were reduced by DFP pretreatment in the case of oxidized nicotine and nicotine-N-oxide but not nicotine. Several auto-oxidation products of nicotine were tested for tremor and/or convulsion-potentiation by DFP pretreatment. Only with nicotine-N-oxide was potentiation observed. The anticholinesterase potentiating effects observed are believed to be due to the specific nicotine-N-oxide, nicotine-1'-oxide. "Free" and "bound" acetylcholine levels were measured in mice treated with nicotine-N-oxide with and without DFP pretreatment. No change in these levels were produced by

nicotine-N-oxide alone. DFP pretreatment resulted in a significant two-fold increase of the total brain acetylcholine measured with the highest proportional increase occurring in the "Bound" fraction. In DFP treated mice, nicotine-N-oxide and nicotine caused a significant reduction in the "bound" acetylcholine and a significant rise in the "free" acetylcholine recovered. No qualitative difference in this effect was demonstrated between nicotine and nicotine-N-oxide. The mobilization of "free" acetylcholine from the high "bound" fraction in DFP treated mice is thought to be the reason for the potentiation of the nicotine-N-oxide tremor. This is based upon the indirect pharmacological evidence obtained from the effects of atropine, mecamylamine and hemicholinium-3. This effect of tremor and/or convulsion potentiation by brain acetylcholinesterase depression was not reflected in the levels of "free" acetylcholine measured; however, the uniform reduction of the "bound" fraction of acetylcholine following treatment with nicotine or nicotine-N-oxide indicated a mobilization of acetylcholine from its "bound" form. The reason for the tremorogenic effects of nicotine-N-oxide being potentiated in DFP treated mice, yet not nicotine, is attributed to the great difference in potency between these two compounds as direct acting nicotinic agonists. The tremorogenic muscarinic component of action of both of these compounds, due to their ability to mobilize acetylcholine, is being masked in the presence of the more potent compound nicotine.

1. INTRODUCTION

The first interest in research pertaining to this study was initiated by the author in work which was done at the Defence Research Establishment Suffield. At this time it was observed that mice which were treated with one-half the lethal dose of the nerve gas VX were six times more sensitive to the tremorogenic effect of nicotine than mice which were not treated with this agent. This was looked upon with interest in view of results in the literature concerning the effects of cholinesterase inhibition upon nicotine. Eccles, Fatt and Koketsu (1954), demonstrated that cholinesterase inhibition was able to potentiate the effect of acetylcholine (Ach) at the central cholinergic "nicotinic" synapse of the Renshaw cell. Curtis and Eccles (1958b), reported however, that the effect of nicotine at this synapse was not similarly affected by cholinesterase inhibition.

When the preliminary observations had been made by the author, it was noted that the sample of nicotine which was used was very dark in color and obviously oxidized from storage on the laboratory bench at room temperature for an undetermined period of time.

Interest was maintained in this finding and upon arrival at the University of Western Ontario the subject was investigated more

intensively. Here however, diisopropyl phosphonofluoridate (DFP)¹ and pinacolyl methylphosphonofluoridate (Soman)² were used as the inhibitors of cholinesterase and the initial studies were carried out using pure nicotine as nicotine bitartrate salt³ or fresh alkaloidal nicotine⁴, clear in color and stored under refrigeration. In these instances, the previously observed potentiation of the tremorogenic effects of nicotine by the anticholinesterase pretreatment was not observed. When a sample of naturally auto-oxidized nicotine⁵, dark brown in color, was used however, the phenomenon of tremor and/or convulsion potentiation was repeated. The author believes that the cholinesterase inhibitors which were used to demonstrate this effect were acting in a similar manner to inhibit brain acetylcholinesterase, thus the potentiating effect observed was thought to be due to a product or products, in the oxidized nicotine which were not present in the pure samples of nicotine. It is upon this premise that the present investigation was founded.

¹ Aldrich Chemical Company

² Defence Research Establishment Ottawa

³ The British Drug Houses Ltd.

⁴ Eastman Chemical Company

⁵ Auto-oxidized nicotine refers to oxidized nicotine which had taken place spontaneously over a period of greater than two years of storage at room temperature in the presence of air and light

II. HISTORICAL REVIEW

A. Nicotine as a Centrally Active Cholinomimetic

Nicotine has been studied for its own action and its ability to mimic the effects of Ach for many years. The term "nicotinic" action of Ach was introduced by Dale (1914) to describe the action of Ach on peripheral ganglia which was not blocked by atropine but which could be blocked by an excess dose of nicotine. After the demonstration by Loewi (1921) of the role of chemical mediation in nervous transmission and later when, Loewi and Navratil (1926) reported that Ach was indeed such a transmitter the drug nicotine has been used extensively to demonstrate the "nicotinic" action of Ach and classify cholinergically mediated synapses as being either "nicotinic" or "muscarinic". Langley and Dickinson (1889) noted the dual action of nicotine on autonomic synapses. This effect was first to stimulate when applied at a low dose, then to produce a block at a higher dose. They used the blocking action of nicotine to demonstrate the association of the peripheral ganglia with a number of autonomic functions.

The positive indication of a nicotinic cholinergic process of nervous transmission in the central nervous system appeared with the work of Eccles et al (1954), and Curtis and Eccles (1958a, b).

According to Holmstedt and Lundgren (1967), Van Praag (1885) noticed the tremor induced in animals poisoned by nicotine, but directed his attention to the effect of the compound on skeletal muscle. There are a number of reports in the literature which describe the tremor produced by nicotine as being centrally mediated with the dose response for tremor, clonic convulsions, tonic convulsions and death being established in several species (Bium and Zacks, 1958; Stone, Meckelnburg and Torchiana, 1958; Arutyunyan and Mashkovski, 1961; Day and Yen, 1962; Orcutt, Michaelson and Prytherch, 1963; and Yamamoto, Orori and Inok, 1966). The electrophysiological response associated with nicotine induced tremor and seizures has been described by numerous authors. Much of this work is summarized by Silvette, Hoff, Larson and Hoag (1962). Here it is reported that nicotine produces a typical "grand mal" seizure pattern on the EEG at large doses. Doses just below that required for the seizure pattern produce only prolonged desynchronization. It is reported that the desynchronization effect is probably due to excitation of the reticular formation while the convulsive effect is produced by excessive excitation probably involving cortical neurones.

Auto-oxidation Products of Nicotine

Wada, Kisaki and Saito (1959) studied the auto-oxidation products produced when air was bubbled through nicotine for four weeks at 30° C. Nicotinic acid, nicotine, cotinine, myosmine and nicotine-N-oxide were all present as air oxidation products with nicotine-N-oxide being present in the largest quantity; i.e. 16 per cent of the original nicotine was recovered as this substance while

of the other identified compounds found, nicotinic acid was present in the second greatest amount at only 0.45 per cent. Reports of studies conducted on the metabolism of nicotine in vivo by Papadopoulos and Kintzios (1963), Papadopoulos (1964) and Hansson and Schmitterlow (1964) revealed that nicotine was changed in the body to cotinine, demethylcotinine, nornicotine and nicotine-1'-oxide. Schmitterlow, Hansson, Andersson and Appelgren (1967) in reporting on the distribution of nicotine in the central nervous system showed that although there was a rapid accumulation of nicotine in the brain of the mouse after intravenous administration it is unlikely that any of it is metabolized in this area. Only do metabolites such as cotinine appear in the brain after sufficient time has elapsed for the product to be formed by oxidation in the liver and translocated to the brain. It is reported by Bizard, Vanlerenberghe and Lespagnol (1956); Larson, Hoag and Silvette (1961); Arutyunyan and Mashkovski (1961); and Clark, Rand and Vanov (1965) that none of the oxidative metabolites of nicotine are more biologically active than nicotine itself. In the knowledge of this it thus becomes desirable to look for an alternate action of nicotine oxides to explain the observation prompting this study.

Nicotine and Acetylcholine Release

It has been suggested that nicotine may act in the central nervous system by mimicking the effects of Ach or possibly that of causing Ach release (Knapp and Domino, 1962; Ginzel, 1967). There have been a number of studies which support the idea that one of the actions of nicotine in the central nervous system may be that of causing Ach release (Armitage, Milton and Morrison, 1966; Armitage and Hall, 1967b;

Morrison, 1968; Corley and Hoff, 1969; and Morrison, Goodyear and Sellers, 1969). This evidence has been mainly based upon the potentiating effect which eserine had upon the particular nicotine response being measured. A recent study by Armitage, Hall and Sellers (1969) demonstrated that small doses of nicotine will cause an increased release of Ach from the cerebral cortex.

Evidence for a similar mode of action of nicotinic agents in the periphery has been reported. Day and Vane (1963); Takagi, Takayanagi, Irikura and Nishino (1967); and Henderson, Ariens and Simons (1968) have presented evidence to support an action of nicotine, choline and tetramethylammonium (TMA) on smooth muscle by the indirect effect of releasing Ach. This was reported in relation to the effect of these compounds on the guinea pig ileum. Chiou and Long (1969) described the Ach releasing effects of the nicotinic agents tetramethylammonium (TMA), decamethonium (C_{10}), vinylcholine ether and 1,1-dimethyl-4-phenylpiperazinium (DMPP) on the biventer cervicis muscle of the chick. These latter authors also showed that the effects of nicotine, DMPP, a low dose of decamethonium, trimethylamino pentanol, trimethylamino hexanol and choline were all potentiated by treating this muscle preparation with eserine. This they ascribed to the Ach releasing property of these compounds.

Since it is upon this premise that nicotine and/or one of its oxidation products may cause Ach release in the central nervous system (CNS), a review of the knowledge concerning the storage of Ach in the CNS and ways in which its storage may be affected will form the remainder of this literature review.

B. Acetylcholine Binding in the Central Nervous System

General Historical Remarks

The idea that Ach may exist in the living animal in a form where it is incapable of taking part in a biological reaction and also protected from enzymatic hydrolysis by cholinesterase has been known for considerable time. In 1933 Chang and Gaddum suggested that Ach was stored in special structures which prevented it from freely diffusing throughout the tissue. Beznak (1934) proposed that Ach existed in the form of a very labile "precursor" and that this precursor was thought to give off Ach easily upon nervous stimulation. A combined form of Ach in brain tissue was also described as a "acetylcholine precursor" by Mann, Tennenbaum and Quastel (1938). The indication that Ach could be bound in the blood, rendering it biologically inactive and protected from enzyme hydrolysis came from the work of Broun and Scheiner (1935) and Kahne and Levy (1938). Corteggiani, Gautrelet, Kaswin and Mentzer (1936a) made the distinction between Ach existing as either a "free" form or a "bound" complex. They reported that the bound complex is able to liberate Ach into the free form. These same authors (1936b) showed that the yield of free Ach increased when a bound extract was heated. Their definition as to free Ach is that of biologically active Ach which is susceptible to hydrolysis by cholinesterase. Corteggiani (1937) similarly showed that heating increased the yield of free Ach obtained from extracts of the central nervous system. The effect of organic solvents and acidic conditions were shown to produce a similar effect in freeing bound Ach by Mann, et al (1938). Loewi, Hagen, Kohn and Singer (1938) also demonstrated that a Ringer insoluble residue from extracts of

the central nervous system which were not attacked by esterases could release Ach when treated with acidified ethanol or strong acid.

Free and Bound Acetylcholine Within the Cell

In the work conducted by Mann et al (1938), an association was made between bound Ach and cellular tissue. It was observed by Trethewie (1938) that cell free extracts did not show an increase in Ach upon heating thus they suggested that a cellular type of binding was involved. Abdon and Hammarskjold (1944) later showed however, that extracts from organs which were removed, frozen and then extracted, free from cells and protein also showed no free Ach, but when such extracts were acidified and heated or treated with trichloroacetic acid, free Ach was recovered. Since these extracts were thought to be free of protein, the Ach recovered was believed to be due to a breakdown of a labile complex rather than Ach synthesis. These authors termed this complex a precursor of Ach and made the assumption that there is no preformed free Ach present in the tissue at all but rather it is all derived from this labile precursor. Welsh and Prajmovsky (1947) attempted to repeat some of the experiments done by Abdon and Hammarskjold using nervous tissue. The results which they obtained were inconclusive, however in some of their experiments conducted on cell free extracts they were able to obtain an acetone precipitate which showed high Ach activity after boiling. These authors showed that freezing and thawing of the tissue had an effect upon the amount of Ach recovered and that also Ach synthesis in the extract could complicate obtaining reliable readings on the amount of recoverable Ach obtained.

Feldberg (1945b) accepted the view that there was a fixed

Ach content of tissue under normal physiological conditions, the level of which may be determined by the number of available cell constituents to which the Ach may be linked. He also followed the view previously advanced in stating "there is no evidence that 'free' acetylcholine exists or can exist in tissue". His suggestion was that tissue particles maintain their normal store of Ach and replace it as liberated, if synthesis is operating in a normal fashion. These stores were said to be unable to build up beyond their normal physiological level.

More extensive measurement of free and bound Ach in the central nervous system was conducted by Elliott and Henderson (1951) investigating these levels in the cerebral hemispheres of rats. Their criterion to define free Ach is that which could be eluted by a Leech-Ringer phosphate medium at pH 7.2 containing eserine. The total Ach of the tissue was that which could be extracted by the same medium at pH 2.2 for one hour. Bound Ach was then determined from the difference between the level of these two readings. These authors found a great deal of variability in the amount of free Ach which they extracted. They suggested this could have been due to the physical factors concerned with the extraction procedure such as freezing of the tissue prior to extraction. These authors also referred to the free Ach which they measured as largely an artifact. Even though freezing and thawing of the tissue has been shown to cause a change in the amount of free Ach recovered in relation to the amount of bound Ach remaining in the tissue; Crossland (1951) indicated that higher total recoverable yields of Ach were obtained from whole brains after freezing than when they were not frozen. Stone (1955) investigated many of the problems encountered in attempting to measure the components of free and bound

Ach in the brain. Evidence was presented to show that freezing and thawing of the tissue caused a release of free Ach from the bound form and also that synthesis of Ach continuing after brain homogenization would result in higher than actual amounts of free Ach recovered. Stone reported that the major portion of the bound Ach was associated with the insoluble cellular residue obtained after homogenization and centrifugation, however a small amount of bound Ach was found in the supernatant fluid. This author suggests that this latter fraction of Ach may be complexed with soluble protein or possible lipids. The reported ease with which the insoluble bound Ach was released introduced the notion that a chemical type of binding was doubted and that the Ach was more likely bound within a structure. A similar hypothesis had been advanced previously by Brodtkin and Elliott (1953) where the structure possibly involved was suggested to be that of the mitochondria. These structures were known to be susceptible to osmotic disruption and this process was also found to free Ach from its bound form.

Subcellular Structural Confinement of Acetylcholine

Intensive investigation on the subcellular structures in the region of the neuronal synapse revealed the presence of organelles which were found to be associated with Ach storage and release. De Robertis and Bennett (1955) isolated subcellular fractions from nervous tissue and by electron microscopy identified a granular or vesicular component on the presynaptic side. These structures were identified as synaptic vesicles. These vesicles were found to be closely associated with the presynaptic membrane and electron micrograph observations indicated what appeared to be some of the

vesicles extending through perforations or gaps in the presynaptic membrane. This suggested the vesicles had a function associated with transport across the membrane. De Robertis and Franchi (1956) observed the vesicles associated with the rod and cone synapses of the eye in rabbits and obtained further evidence for their involvement in a functional role. Rabbits which had been dark adapted for a period of 24 hours showed that the synaptic vesicles were concentrated around the presynaptic membrane. After a prolonged exposure of rabbits to darkness (9 days), the size of the vesicles observed was found to have decreased. Robertson (1956) also observed vesicles in the area of the terminal axoplasm at the myoneural junction. In this study the possibility is raised that the structures appearing as vesicles may have actually been tubules in cross section, however this author does draw an association between the observed synaptic vesicles and the "quantal" release of Ach which had been proposed by del Castillo and Katz (1954). Robertson (1956) advances the suggestion that these vesicles may represent the prejunctional, subcellular, morphological units of neurohumoral discharge.

With the identification of the synaptic vesicles and their implication in the role of chemical transmission, further observations were conducted in attempting to correlate these structures with a cholinergic functional role. Hebb and Whittaker (1958) demonstrated the presence of Ach in a subcellular particulate fraction found in the same fraction as the mitochondria. They demonstrated that 50 per cent of this particulate bound Ach was released with mild changes in tonicity of the suspending medium or by freezing and thawing. These authors implied the presence of Ach and choline acetylase as being present in

the same cellular particle. Bellamy (1959) agreed with these latter findings in that he found choline acetylase and bound Ach were both located in the separation fraction containing mitochondria. In this investigation extracts were made from rat and pigeon brains using differential centrifugation and 70 per cent and 54 per cent respectively of the total bound brain Ach was found to occur in this fraction.

Whittaker (1959) prepared a brain fraction of synaptic vesicles which was more pure than any which had been examined to this time in that it was devoid of mitochondria. This fraction was found to contain mostly particle bound Ach, and electron microscopic examination of the fraction showed it to contain organelles appearing as simple vesicles. This work demonstrated the existence of two bound forms of Ach, each representing 50 per cent of the total bound Ach. One form was found to be more labile than the other in that it could release Ach upon mild treatment (i.e. hypotonicity of the suspending medium or freezing and thawing); while the other bound form could only release Ach upon incubation at 37° C, exposure to pH values of 4.0 or less or by treating the extract with organic solvents. Whittaker suggested that the binding involved in the more stable fraction was by chemical forces whereas the more labile fraction seemed to be contained only by structural envelopment. Work which was to follow indicated the main difference in all probability lies in the differential structural integrity of the membrane enveloping the two Ach pools, as well as the possibility of different forces of chemical binding. Gray and Whittaker (1960, 1962) using density gradient ultracentrifugation and electron microscope identification, demonstrated that the fraction rich in bound Ach consisted mostly of pinched off nerve endings which they

called "synaptosomes". Whittaker, Michaelson and Kirkland (1964) conducted a more critical separation of subcellular particles from brain tissue and obtained a more purified fraction of synaptic vesicles. This was done by an increase in the refinement of their density gradient ultracentrifugation technique. Two fractions, rich in Ach, were thus separated. One fraction was found to contain entirely synaptic vesicles and the other fraction contained intact synaptosomes, as identified by electron microscopy. From their study they concluded that the synaptic vesicles are the ultimate storage sites of Ach within the cholinergic nerve ending and that Ach is not synthesized within the vesicle but rather in the cytoplasm of the synaptosome. Whittaker and Dowe (1964) produced positive evidence that the Ach like substance in the synaptosomes derived from guinea pig brain was in fact Ach itself. Their technique consisted of separation of Ach from the brain using column chromatography which was capable of differentiating Ach from analogues such as acetylcarnitine and propionylcholine. Ryall, Stone and Watkins (1964) also presented strong arguments for the cholinomimetic extract in nerve terminals being none other than Ach. Their evidence was based upon parallel biological assays performed before and after the purification of their brain extracts by electrophoresis and chromatography.

Takeno, Nishio and Yanagiga (1969) separated out two distinct bound fractions of Ach from the synaptosomes. The extraction procedure to rupture the synaptosomes, used by these authors, was different than that carried on before which had been based upon hypotonic treatment after Whittaker (1964). These more recent authors made use of the French press technique which they report did not rupture any of the

synaptic vesicles and completely ruptured the synaptosome membranes. They reported that the previously used hypotonic treatment was unsuccessful in rupturing all synaptosomes while at the same time caused some of the synaptic vesicles to rupture. Their evidence for this occurrence with hypotonic treatment comes from electron micrographs showing the presence of phosphotungstic acid inside some of the vesicles, indicating them to have ruptured, and outside some of the synaptosomes indicating them to be still intact. As a result of this different technique of subcellular fractionation, Takeno et al (1969) found a higher yield of bound Ach associated with the vesicular fraction than previously reported. They made the distinction that the stability of the two bound fractions is not the same as that reported by Whittaker (1964), which had been categorized by hypotonic treatment. The stable bound fraction defined by Takeno et al (1969) could only be turned into free Ach by drastic treatment (pH 4.0 at 100° C for 10 minutes or shaking with ether and freeze drying). The labile bound fraction is suggested to be in the synaptosome cytoplasm outside of the vesicle membrane. The two fractions are thus separated on the basis of being mechanically labile and stabile. These authors denote a difference in the properties of the vesicular membrane as compared to the synaptosomal membrane in their ability to confine their compartmentalized Ach. A difference in the two bound fractions to release Ach was noted in that the intact vesicles do not lose any of their Ach following incubation for 24 hours in 0.32 M sucrose, however, the intact synaptosomes incubated in this same medium lose 50 per cent of their bound Ach over this same time period.

Whittaker (1965) defined three "compartments" for Ach in the cholinergic neurone. These are:

1. Ach present in the cell body and axon
2. Ach present in the synaptosomal cytoplasm
3. Ach present in the synaptic vesicles

The present state of knowledge regarding the binding of Ach and its subcellular depots allows one to diagrammatically represent the locations of the different forms of Ach in the nerve terminal. This is illustrated in Figure 1.

Beani, Bianchi, Megazzini, Ballotti and Bernardi (1969) reviewed the recent concepts as to how the three storage pools of Ach were related, i.e. free Ach, labile bound Ach, and stabile bound Ach. It is presently believed that Ach is synthesized in the cytoplasm where it may be integrated into the vesicular stores. The vesicular pool is the one which is actively involved in Ach release. Upon release of the Ach in the vesicles, it may act on the post synaptic membrane receptor and undergo hydrolysis by cholinesterase. Choline may then be re-taken up into the synaptosome to provide substrate for resynthesis. The labile bound pool of Ach may take up free Ach from the synthesis which occurs in the cytoplasm. It has been shown that the rate of synthesis may be influenced by the facility for Ach storage and the amount of Ach in any one of these three pools may depend upon the rate of firing of the neurone or the effect of drugs upon synthesis, release or destruction of Ach.

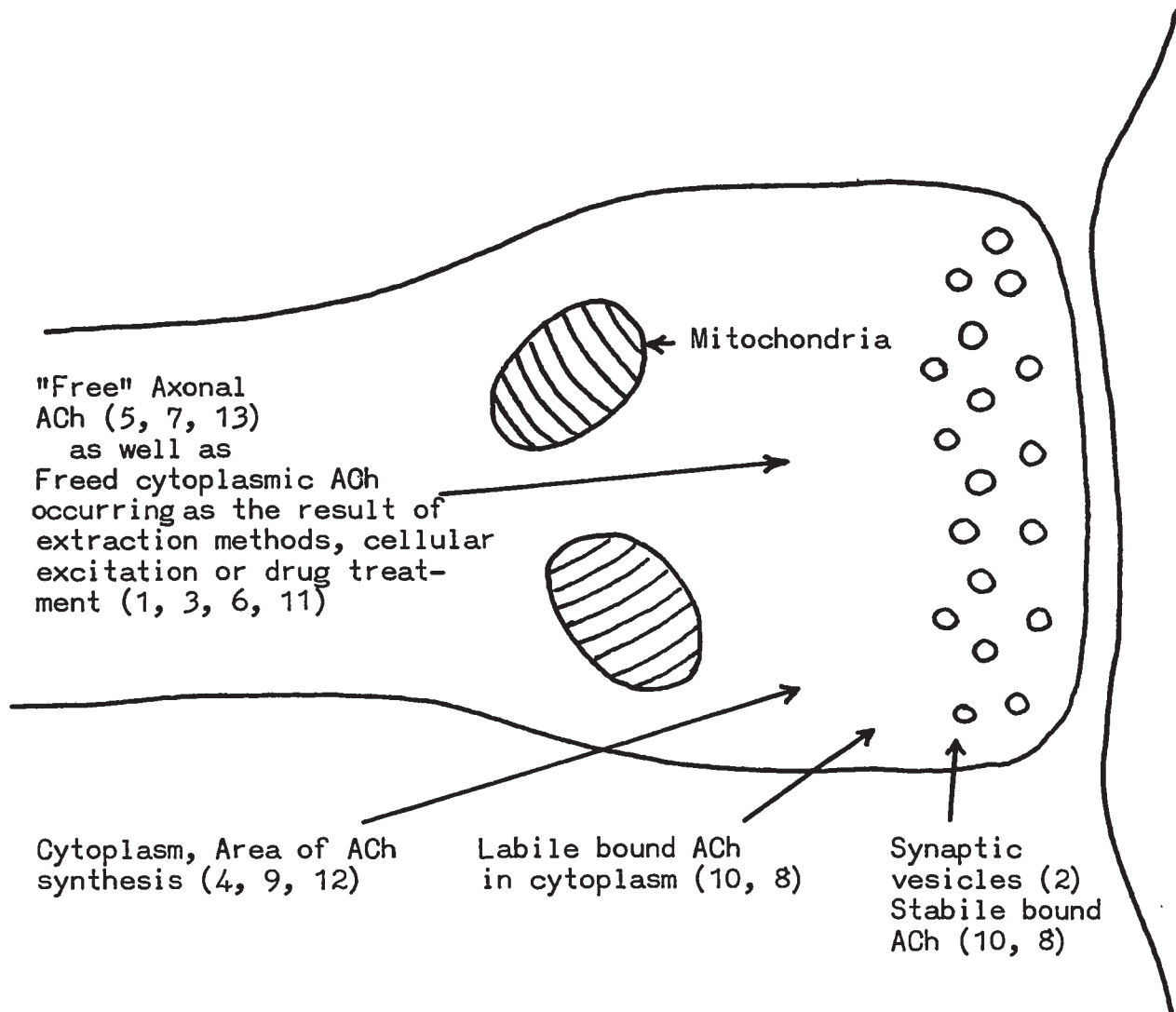
Subcellular Synthesis and Uptake of Acetylcholine

Evidence gathered to date most favors the idea that Ach is synthesized within the cytoplasm of the nerve ending, and then

Figure 1

Diagrammatic Representation of the
Cholinergic Nerve Ending Showing
the Regions of Acetylcholine Deposition¹

¹the numbers in the diagram refer to the authors
cited in the figure.



- | | | |
|------|-------------------------|--------|
| (1) | Abdon and Hammarskjöld | (1944) |
| (2) | De Robertis and Bennett | (1955) |
| (3) | Elliott and Henderson | (1951) |
| (4) | Gray and Whittaker | (1962) |
| (5) | Hebb | (1963) |
| (6) | Hemsworth and Neal | (1968) |
| (7) | MacIntosh | (1941) |
| (8) | Marchbanks | (1967) |
| (9) | Michaelson | (1967) |
| (10) | Takeno et al | (1969) |
| (11) | Welsh and Prajmovsky | (1947) |
| (12) | Whittaker et al | (1964) |
| (13) | Whittaker | (1965) |

incorporated in some fashion into the synaptic vesicles (Michaelson, 1967; Whittaker et al, 1964). This concept accepts that the enzyme of synthesis, choline acetylase is located in the soluble cytoplasmic component while the hydrolytic enzyme acetylcholinesterase is found bound to the microsomal membranes. There has been some controversy over the presence of choline acetylase being only in the soluble portion of the synaptosome. De Robertis, de Iraldi, de Lores Arnaiz and Salganicoff (1962, De Robertis, Salganicoff, Zieher and de Lores Arnaiz (1963), McCaman, de Lores Arnaiz and De Robertis (1965) and Saelens and Potter (1966) all indicate the presence of this enzyme in the synaptic vesicles. Michaelson (1967) discusses this controversy and presents the argument for choline acetylase being present only in the soluble portion of the cytoplasm at normal ionic strength and pH. Differences in these external factors encountered during the previously reported extraction procedures could alter the permeability of the vesicular membrane. Work done by Tucek (1966) also does not support the idea that choline acetylase is located in the synaptic vesicles, however, he did point out that there was a species difference in the ease with which this enzyme was extractable.

Bellamy (1959) demonstrated that Ach synthesis was not operating continuously in the subcellular mitochondrial fraction and it was suggested that the choline acetylase enzyme and choline were not the limiting factors on the rate of synthesis as they both were found to be present in relatively large amounts. A parallelism between the rate of synthesis and the capacity of the brain particles to bind and release Ach was indicated. This suggested a feedback

mechanism between the amount of Ach in the bound or free form and the rate of synthesis. When the particulate fraction containing bound Ach was incubated with choline, no increased synthesis of Ach was indicated, however, this addition of choline brought about a release of Ach from the particle bound state. This likely occurs in a similar fashion to the exchange mechanism suggested by the early work of Renshaw, Green and Ziff (1938) who demonstrated that choline and choline analogs would bring about a liberation of Ach, and is perhaps the reason why Mann et al (1938) found that conditions which favored increased Ach synthesis (high choline) increased the fraction of free Ach recovered. Giarman and Pepeu (1962) made the suggestion that the concentration of Ach in the vicinity of the site of synthesis regulated the rate of Ach formation. Hubbard and Kwanbunbumpen (1968) indicated that the specific synaptic vesicle population in the pre-synaptic region forms part of a feedback mechanism adjusting transmitter synthesis and mobilization to the rate of release of the transmitter. Sharkawi and Schulman (1969) supported the suggestion made by Giarman and Pepeu (1962) by presenting evidence to show that the concentration of Ach in the vicinity of the sites of synthesis regulates the rate of Ach formation. Decreased levels of Ach in the tissue enhanced the rate of synthesis while increased levels had a depressing effect.

It thus remains a question as to how the newly synthesized Ach is incorporated within the synaptic vesicles as there is yet no satisfactory explanation as to how this takes place. Burton and Howard (1967) advance a theory whereby gangliosides play an important role in the structural formation of the synaptic vesicle and the binding of Ach. They picture the vesicle as being composed of a

protein matrix whose "holes" are filled with lipids, including gangliosides. In aqueous solution such as the cytoplasm, they exist as large aggregates. Upon depolarization of the pre-synaptic membrane they come in contact with the lipid portion of the membrane. It is suggested that then the ganglioside portion of the vesicle deaggregates and dissolves in the lipid membrane and other lipid components of the vesicle, like phospholipids and cholesterol, do the same. The polar Ach is then forced through the lipid membrane and expelled into the synaptic cleft. With repolarization of the pre-synaptic membrane the protein matrix of the vesicle membrane is repelled away, back into the cytoplasm and cytoplasmic gangliosides are taken up to reform vesicles. Burton and Howard (1967) state that the vesicles are able to take up Ach from the cytoplasm and in addition synthesize it within itself. As there is conflicting evidence in the literature concerning these two premises perhaps their theory could be reconciled on the basis that Ach is bound to the lipid fraction of the vesicle before or just at the time the vesicular structure is reformed. The work of Kuriyama, Roberts and Vos (1968) and that of Guth (1969) also presents evidence that the synaptic vesicles are able to take up Ach. Marchbanks (1967) challenges this assumption, with his criticism of work done previous to his time being based upon the methods which were used. His results emphasize the need for absolute certainty that the population of synaptic vesicles one is working with is free from synaptosomes. He made use of gel filtration techniques for the separation of the subcellular components. Marchbanks' results indicated that in no case did his fraction of vesicles take up Ach in excess of 5 per cent and in most cases it was below 2 percent. This

author does, however, support the idea that Ach exists in two bound forms within the synaptosome. One form being a labile bound component which is identified with the cytoplasmic Ach and the other is a stable bound form of non-exchangeable Ach contained within the synaptic vesicles.

Acetylcholine Binding and its Relationship to Synaptic Transmission

Whittaker (1965) reviewed the evidence which reconciles the vesicles as the ultimate storage sites of Ach associated with the quantum hypothesis of Ach release. Hubbard and Kwanbunbumpen (1968) presented more recent evidence for this association as well. In their work, high levels of KCl, which increased the quantal release at the neuromuscular junction also produced an accompanying decrease in the number of synaptic vesicles adjacent to the nerve terminal membrane. Whittaker (1965) expressed confidence that the theoretical calculations of the Ach content of the synaptic vesicles is consistent with the quantum hypothesis of Ach release. With respect to Ach release, two suggestions were advanced at this time as to the possible mechanism involved. These were:

- synaptic vesicles fuse with the external pre-synaptic membrane and subsequently discharge their transmitter contents by a process similar to reverse micropinocytosis.
- upon nervous stimulation the vesicles form a "tubule" system for massive rapid transmitter release.

It is to be recognized that the theoretical calculations of vesicle content and its relation to the quantum hypothesis of Ach release suffers from many drawbacks. It is thought advisable that these be pointed out in this review.

It is necessary to keep in mind that only a certain percentage of the vesicles in the homogeneous population of vesicles which have been examined may have come from cholinergic neurones and thus may be more likely to contain Ach. This will vary greatly depending upon the area of the brain from which the population of neurones were drawn, i.e. approximately 20 per cent of the neurones examined by micro methods in the cerebral cortex were found to be cholinceptive by Krnjevic and Whittaker (1965) and up to 58 per cent of the brain stem neurones are reported to be cholinceptive by Bradley and Wolstencroft (1967).

Cholinceptivity of a neurone is not to be taken as being synonymous with cholinergic innervation as argued by Karczmar (1969).

Estimates of the size of the vesicles used to obtain a best estimate of their internal core diameter are reported to vary. From the observations of de Robertis and Franchi (1956) it was indicated that the size of the vesicles could vary as a function of nervous activity.

Electron micrographs of the subcellular particles from cerebellum nerve terminals has indicated the presence of vesicles differing both in size and shape according to the work of Larramendi, Fickenscher and Lemkey-Johnson (1967), and Uchizono (1965). Some attempts have been made to isolate them, one from the other on the basis of their biochemical differences by Vos, Kuriyama and Roberts (1968) but so far without any success. These authors as well as a separate study by Kuriyama, Roberts and Vos (1968) have shown synaptic vesicles to be capable of binding γ amino butyric acid, Ach, norepinephrine and 5-hydroxytryptamine.

The type of binding within the discrete internal core of the vesicle would influence the molecular content. Canepa (1964) reports on the possibility of solid-state forces in operation and that Ach must be bound in a crystalline form, if one is to allow for as much as 1×10^5 molecules being present in one vesicle.

Correlations of the contents of the vesicle with the number of Ach molecules per quantum are based upon experiments conducted at the neuromuscular junction. To mention the extremes in variability between values thus derived they range from 900 molecules according to MacIntosh (1959), to 100,000 molecules according to Krnjevic and Mitchell (1961).

In spite of the present inability to positively identify synaptic vesicles which contain only Ach, or that a cholinergic neurone contains only vesicles containing Ach, it does appear certain that Ach is bound within the confines of the synaptic vesicle and that this in all likelihood is the functional unit of Ach discharge.

Chemical Aspects of Acetylcholine Binding

Throughout the studies conducted concerning the binding of Ach, investigations have been carried out upon the chemical nature of this phenomenon. This subject is extensively reviewed by Green (1962) and many of the references cited prior to this date are covered in his work.

Early evidence by Chang and Gaddum (1933) indicated that the Ach they extracted may have been bound to lipids as well as protein, as they found that alcoholic extracts of tissue removed less Ach than trichloroacetic acid extraction. Barsoum (1935) and also Loewi and Hellauer (1938), indicated that this difference could be

accounted for when the residue from the alcoholic extraction was then further extracted with trichloroacetic acid. Additional indication that lipids may be binding Ach in neural tissue comes from the finding that ether extracts of nervous tissue contained Ach (Feldberg, 1945a; and Loewi, 1956). Foch, Lees and Sloane-Stanley (1957) and Foch (1959) indicated that ionic potassium could displace some of the Ach like material from a lipid extract of nervous tissue and that the reaction of drugs may similarly be able to release any cationic amine bound to the lipid. This was observed in that choline could exchange with inorganic cations which were bound to acid lipids. Goldberg (1961) found that cerebroside sulfate, could complex with many biogenic amines, among them being Ach. Ach was also found to combine with a phosphatide like material by Woolley (1959). Green (1962) in his review, criticizes these findings which implicate lipids as the materials Ach is bound to in vivo as these previous experiments were carried out in vitro and the lipids which had been suggested are known to already be in an associated form in vivo.

The idea that Ach may bind with proteins has been long held for it is the concept that the receptor for biogenic amines is protein in nature. It is pointed out by Ehrenpreis (1960) that this "receptor" protein shows affinity for both cholinergic activating and cholinergic inhibiting substances.

Nachmansohn (1959) reported that many potential substances may bind with Ach such as polysaccharides, nucleic acids and non-specific proteins. These all may form complexes with positively charged materials. Green (1962) indicated that because of the ease of removal of much of the bound Ach, as referred to earlier in this review

under the heading of subcellular structural confinement of Ach; it is thought that a portion of the bound Ach is loosely linked to tissue. For example, by ionic or hydrogen bonding. Osawa, Imai and Kagawa (1954) indicated that electrostatic bonding is the prime means of binding substances to polysaccharides, proteins, nucleic acids and perhaps some lipids. This being so, organic and inorganic cations may compete for these anionic sites and mutually displace one another. Burton (1964) and Burton and Howard (1967) have been strong supporters of the role of a glycolipid ganglioside in the binding of Ach. Burton (1964) has demonstrated that the subcellular distribution of Ach and gangliosides are parallel in the rat brain and that the synaptic vesicle fraction is rich in both Ach and ganglioside. Agents which affect gangliosides also promote the release of Ach from its bound form. Kuriyama et al (1968) offer the suggestion that gangliosides, sialoproteins, hyaluronic acid and chondroitin sulfate could possibly furnish the anionic binding sites associated with the synaptic vesicles. These latter authors have shown that treatment of the vesicular particles with neuraminidase removed a total of 68 per cent of the N-acetylneuramic acid from the vesicles and decreased the binding capacity of the vesicles for Ach. They suggest that one of the attributes of Ach binding sites on the vesicles is the presence of exposed, highly ionized, polyanionic constituents; as they found the most effective inhibitors of Ach binding were the polyamines, spermine and spermidine.

Drug Effects Upon Acetylcholine Release and Binding

It has been pointed out by MacIntosh (1961) that drugs which have a specific action towards cholinergic synapses may exert

their effect in a number of the following ways:

Pre-synaptically - by affecting the transport or acetylation of choline

- by affecting the storage and release of Ach

Post-synaptically - by mimicking or blocking the action of released Ach

Either post-synaptically or pre-synaptically - They may affect the enzymatic or non-enzymatic removal of Ach.

This discussion is to be limited to drugs which affect the storage and release of Ach.

1. Inhibitors of Ach release

There are numerous studies conducted which have shown the effect of drugs or metallic ions on the suppression of Ach release. Harvey (1939) demonstrated that procaine was able to suppress the output of Ach occurring as the result of preganglionic stimulation in the superior cervical ganglion of the cat and also able to suppress this same effect produced by nerve stimulation at the neuromuscular junction. It was later demonstrated by Straughan (1961) that the effects of procaine at the neuromuscular junction were produced by concentrations which were below that thought to be acting by virtue of the curare-like action of the compound. He demonstrated a significant reduction in the amount of Ach released by a period of tetanus at this site in the presence of procaine.

It was shown by Brooks (1956) that botulinum toxin caused a reduction in the frequency of miniature end plate potentials at the neuro-muscular junction and thus ascribed its action to that of affecting Ach release. Simpson (1968) studied the effects of botulinum toxin after i.p. injection in rats and observed a resulting increase in

cortical Ach levels. This was taken as evidence to show that this substance operates in the C.N.S. in a similar manner to its action at the neuro-muscular junction in that it blocks Ach release. The relationship to the blockade of Ach release by botulinum toxin and the quantal association of Ach with the synaptic vesicles is not clear as it has been shown by Thesleff (1960) that after botulinus intoxication there was no apparent change in the population of synaptic vesicles observed. Fleisher, Killos and Harrison (1961) demonstrated a reduction in Ach output at the neuromuscular junction as the result of exposure to puffer fish poison and Ogura (1966) showed the inhibiting effect of tetrodotoxin on the release of Ach in the transmurally stimulated guinea pig ileum. A pre-ganglionic effect, due to the reduction in Ach release is suggested for paraldehyde and methylpentynol by Quilliam (1959) and methylpentanol by Marley and Paton (1959). Ethanol is also reported to decrease the amount of Ach released from unstimulated rat cerebral cortex by Kalant, Israel and Mahon (1967). This effect is thought to be due in part to an inhibition of release as it had no effect upon Ach synthesis.

Morphine has been reported to cause a reduction in the release of Ach. Schaumann (1956) and Paton (1957) demonstrated this action regarding the release of Ach from isolated small intestine. Neither of these authors suggest that this occurs as the result of a direct action of this drug on the Ach synthetic mechanism or the stabilization of the bound pool of Ach in the tissue. Green, Carlini and Robinson (1963) detected a lipid extract from sciatic nerve and brain which they suggest caused the release of Ach from the nerve plexus in

the wall of the guinea pig ileum. Morphine was reported to totally block the action of this substance by these authors. Morphine is also reported to depress the rise in Ach output occurring as the result of a neostigmine perfusion of the ventricles of the cat (Beleslin and Polak, 1965; Beleslin, Polak and Sproull, 1965). In addition to the effects demonstrated by morphine in suppressing Ach output, Schubert and Sundwall (1967) have shown that morphine could competitively inhibit Ach uptake in brain tissue slices in vitro.

2. Cellular Ionic Environment and Ach Storage and Release

The balance of ions in the vicinity of the cholinergic synapse has been shown to be important in affecting the storage pools of Ach. Mann, Tennenbaum and Quastel (1939) indicated that incubating brain slices in an eserinizated medium which was high in potassium ions resulted in a decrease in bound Ach. This was later supported by Elliott and Henderson (1951) whose additional work of a similar nature indicated that a low level of bound Ach was associated with a high potassium ion level and high bound Ach with low potassium. Bowers (1967) working with rat cortical brain slices demonstrated the necessity of potassium for the conversion of bound Ach to free Ach. Polak (1969) found as well that the uptake of Ach by slices of rat cerebral cortex was partially inhibited by high potassium levels. It was reported in the work of del Castillo and Katz (1956), Birks and MacIntosh (1957), and Katz (1958) that both potassium and calcium ions were found to enhance Ach release. The necessity for an external supply of calcium for Ach release in studies on the Ach metabolism of the sympathetic ganglia was demonstrated in the work of Harvey and MacIntosh (1940), Hutter and Kostial (1954) and del Castillo and Katz

(1954). Hutter and Kostial (1955) reported that high sodium ion levels were without effect in causing Ach release. The importance of sodium ions for Ach to become bound, however, has been indicated by Quastel (1962) where high levels of sodium ions in the perfusing fluid were necessary for the ganglia to store Ach. Conversely, low levels of sodium caused Ach to leak out into the perfusion fluid. Kuriyama et al (1968) working with a vesicle rich subcellular fraction from the whole mouse brain found that although there was an absolute requirement for sodium ions in the binding of γ aminobutyric acid, the binding of Ach was markedly inhibited by sodium. This may not be a comparable contradiction with the work of Quastel (1962) as these more recent authors were attempting to work with a fraction of cellular tissue rich in synaptic vesicles, and that it has been shown that Ach may also be bound in the synaptosome outside of the vesicles. In a study conducted by Birks and MacIntosh (1961) on the metabolism of Ach in the sympathetic ganglia, it was reported that in addition to the proper ion balance, dissolved CO_2 and an unknown dializable, heat labile material found in the plasma should be present for optimal Ach release.

3. Effects of Drug Induced Excitation and Depression Upon Ach Levels in the Brain

It has been a popular belief that the level of change in Ach content of the brain was a direct function of the drug induced change in brain activity. This is to say that drugs which depressed the central nervous system produced the end result of increasing the brain Ach levels and drugs which acted as C.N.S. excitants caused a decrease in the level of brain Ach. Crossland and Merrick (1954) made this assumption regarding the effect of the anaesthetics they studied and

reported that the fractional increases in Ach concentrations in the brain depended upon the depth of anaesthesia induced. This is a very all inclusive concept and exceptions to this generalization have been found. Bose, Gupta and Sharma (1958) indicated that doses of phenytoin which causes a central depression were accompanied by lower than normal brain Ach levels. Agarwal and Bhargava (1964) similarly found that sedating doses of chlorpromazine did not influence the level of brain Ach in rats. Giarman and Pepeu (1962) although generally following the assumption that central depressants have associated with their action an elevation in total brain Ach, and that a reduction was accompanied with the administration of convulsants; found an exception in that reserpine at a depressing dose caused an initial rise in brain Ach, which was followed by a return to the normal level. This general effect of central depressants and excitants with regard to their effect upon brain Ach is still assumed to be the case as found in the more recent work of Crossland and Slater (1968). Here it was indicated that there were rises in both free and bound brain Ach upon the administration of the anaesthetics pentobarbitone, chloral hydrate, urethane and ether. Phillis (1968) studied the effect of stimulating a variety of peripheral afferent pathways on brain Ach release under the effect of different anaesthetics. Afferent stimulation caused Ach release in all cortical areas in a non-specific manner. This Ach release was reduced under the effects of the following anaesthetics; sodium pentobarbital, Dial, diethylether, halothane and chlorolose, with the effect being less when the volatile anaesthetics were used.

The work of Lundy and Colhoun (1969) give further

evidence to the findings that anaesthesia alone is not the casual factor in the increased levels of Ach which occur, as graded doses of phenylcyclidine derivatives given to rats in sufficient dose levels to cause surgical anaesthesia did not produce an accompanying rise in brain Ach.

It was shown by Hemsworth and Neal (1968) that the centrally stimulating drugs; leptazol, picrotoxin, strychnine, dexamphetamine and nikethamide all stimulated the release of Ach from the rat cerebral cortex. These centrally stimulating drugs tested indicated that their action in enhancing Ach release was approximately parallel to the increase in brain electrical activity they produced. Beleslin et al (1965) also showed an increase in Ach output from the perfused ventricles after the systemic administration of strychnine and leptazol.

There is some evidence indicating that some of these centrally excitant drugs, when administered in the presence of anaesthetic agents, demonstrate a diminished effect upon their ability to increase Ach output. Dudar and Szerb (1968) observed that animals under halothane -N₂O anaesthesia showed a diminished effect of atropine in causing Ach release when it was applied topically to the surface of the brain. It was also shown by Beleslin et al (1965) that amytal anaesthesia reduced the Ach output produced by ventricular perfusion of neostigmine. Amytal showed a greater effect in this regard than chloralose.

In a study conducted by Kurokawa, Michiyama and Kato (1963), a special strain of convulsive mice was used and convulsions were induced by pentamethylenetetrazole in both the convulsive mice and

normal mice. Anaesthetics caused an increase in brain Ach and the convulsant caused a decrease. The greatest difference in the level of compartmentalized Ach occurred in the labile bound fraction. The mice of the convulsive strain contained twice as much bound Ach in this fraction as normal mice. The greatest changes in brain Ach levels were found in this fraction following the administration of pento-barbitone anaesthesia as well as the convulsant pentamethylenetetrazole.

4. Cholinesterase Depression and the Level of Brain Ach

The effects which cholinesterase inhibitors have in causing an increase in brain Ach levels have been known for a long time. This was demonstrated by Cortell, Feldman and Gellhorn (1941) using eserine in the rabbit; DuBois, Doull, Salerno and Coon (1949) with parathion in the rat; Elliott and Henderson (1951) using eserine in the rat; Stewart (1952) and Robinson, Beck, McNamara, Edberg and Wills (1954) using T.E.P.P., DFP and eserine in rats; Michaelis, Finsinger, de Balbain Verster and Erickson (1954) who gave DFP to rabbits; Homstedt and Lundgren (1965) administering F37A (methylisopropoxy-dimethylamino ethylthio phosphine-oxide) to rats and Fonnum and Guttormsen (1969) using DFP administered to rats. All of these investigations with the exception of Michaelis et al (1954) and Fonnum and Guttormsen (1969) were only concerned with the resulting increase in total brain Ach following cholinesterase depression. Michaelis et al (1954) noted that the effects of an intravenous dose of DFP in the rabbit brought about an increase in the level of free Ach released in the cerebral cortex whereas Fonnum and Guttormsen (1969) measured the levels found in the "extra-cellular" fraction as well as that within the synaptosomes.

Slater and Rogers (1968) and Crossland and Slater (1968) studied the components of free and bound Ach from rat brain after treatment with eserine. They noted a total increase in the level of brain Ach as a result of the eserine treatment, with the greatest proportion of the increase being found in the free fraction compared to the bound fraction. Birks and MacIntosh (1961) showed that Ach accumulated above the normal resting level in the ganglion whose cholinesterase had been inhibited, and this additional Ach was not available for release by nervous impulses.

In the work of Fonnum and Guttormsen (1969) the level of synaptosomal Ach was measured following the administration of a dose of DFP sufficient to depress brain cholinesterase to 10 per cent of normal. Even though this anticholinesterase treatment resulted in a 100 per cent increase in total brain Ach only a proportional 15 per cent increase in synaptosomal Ach was measured. These authors ascribe the bulk of the rise in brain Ach to extra-cellular Ach. This finding was unexpected by these authors, however some of the low levels of synaptosomal bound Ach could be due to a function of their methodology. It is to be recalled that Kurokawa et al (1963) reported their greatest drug induced change in Ach levels took place in the "labile bound" fraction. It has been shown by Takeno et al (1969) that this "labile bound" fraction of the synaptosomes may lose Ach upon incubation in 0.32 M sucrose as well as the long known fact referred to earlier that bound Ach may be released into the free form by freezing and thawing of the brain tissue. Fonnum and Guttormsen (1969) allowed the head of the rat, upon decapitation, to fall directly into liquid nitrogen where it remained for a period of 20 seconds.

This procedure according to them only lowers the temperature of the brain to "just above 0° C". They then homogenized the brains in 0.32 M sucrose for extraction of the synaptosomal fraction. It was shown by MacIntosh (1941) that the highest levels of Ach recoverable were in the areas of the basal ganglia, pons and mid-brain. It appears inconceivable that some of these areas, shown to be rich in Ach, would not be frozen by the process of immersion of the head in liquid nitrogen for 20 seconds. If this were to occur it would result in a freeing of Ach from its bound stores in these tissues. A technique described by Takahashi and Aprison (1964) made use of whole animal immersion into liquid nitrogen. They reported that gross observation of dissected rats thus treated revealed that "apparent freezing" of the cortical surfaces of the brains of twenty-eight rats occurred at a critical time of near 10-11 seconds. This occurred when the whole animal was immersed in the liquid nitrogen and not just the head as employed by Fonnum and Guttormsen (1969).

5. Drug-Induced Release of Ach at its Sites of Binding

There is evidence to indicate that some of the cholinesterase inhibitors exert an effect upon the Ach content of the brain tissue in a way which is not directly ascribed to their action as inhibitors of cholinesterase. Schuberth and Sundwall (1967), Polak and Meeuws (1966) and Polak (1969) have shown that eserine inhibited the uptake of Ach in incubated brain cortical slices. Polak (1969) indicated this was also true for the cholinesterase inhibitor AM-1 (O-ethyl S-diethylaminoethyl ethylphosphonothiolate). On the other hand cholinesterase inhibitors like soman, tabun and DFP were found to have no effect in this regard. The suggestion which has been offered

to explain this is that the inhibitors like eserine and AM-1 carry a positively charged group which may react with an anionic binding site, whereas the other cholinesterase inhibitors react with an esteratic site. The compounds binding with the anionic moiety may be competing for sites of Ach binding.

There is much evidence that numerous drugs, other than anticholinesterases, may behave in a similar fashion by competing for anionic binding sites which could otherwise be occupied by Ach. This was first intimated in the work of Renshaw et al (1938) where it was suggested that the vasodepressant action of the choline analogues they studied was due, in part, to the liberation of Ach and they indicated how this could come about through the process of cation exchange. Ziff, Jahn and Renshaw (1938) reported an example of such a phenomenon showing the exchange absorption of ethoxycholine and Ach for cholinesterase. This particular finding demonstrated little physiological significance in itself, however, it did represent a system whereby two similar molecules, having grossly dissimilar biological activities, could compete for the same binding sites.

Aboud (1959) described the binding of anticholinergic drugs, with psychopharmacological action, to the subcellular mitochondrial fraction. This fraction was rich in Ach and subsequently proven to contain the synaptosomes. Similar requirements for tissue binding of these compounds and Ach were hypothesized because of their close molecular similarities. These compounds were thus suggested to have as one of their mechanisms of action, the occupation of binding sites normally occupied by Ach. MacIntosh (1961) demonstrated an effect of hemicholinium -3

concerning the occupation of anionic binding sites which could otherwise be occupied by Ach. Here the indication is made that drugs which act in such a manner may act as inhibitors of Ach synthesis as they prevent the deposition of Ach. A parallelism between the rate of synthesis and the binding capacity of brain particles had been indicated by Bellamy (1959).

The experiments conducted by Bellamy (1959) indicated that when a brain-mitochondrial fraction from pigeon and rat brain was incubated with choline approximately 50 per cent of the bound Ach in this fraction was released. On incubation of the pigeon brain fraction with choline plus acetate, synthesis of Ach took place to restore the initial level of the bound Ach.

Kaita and Goldberg (1969) have presented recent evidence, based upon in vitro studies of Ach synthesis, to show that increased levels of Ach in rat brain homogenates resulted in a corresponding decreased activity of choline acetylase. The possibility of a feedback mechanism between the level of bound Ach in the neuron and the rate of Ach synthesis is suggested.

A type of competition for Ach binding sites was indicated by Bowman and Rand (1961) and MacIntosh (1963) for the drug triethylcholine. It is also indicated that other cholinomimetic drugs may also cause Ach release as shown by the pre-synaptic action of carbachol demonstrated by Volle and Koelle (1961) and McKinstry, Koenig, Koelle and Koelle (1963).

After the demonstration by Abood (1959) that centrally active anticholinergic drugs would bind with the subcellular fraction known to be rich in Ach it was then shown that such drugs could bring

about a lowering of brain Ach; presumably, as previously suggested, by displacing Ach from its sites of bound storage. It was shown that atropine had the effect of lowering total brain Ach levels and that this also occurred with other centrally active cholinolytics (Giarman and Pepeu, 1962 and 1964; and Pepeu 1963). Beani, Bianchi and Megazzini (1964) indicated that the changes in brain Ach in guinea pig brains after the administration of scopolamine were restricted to specific areas. Lowering occurred in the olfactory lobes, parietal cortex and caudate nucleus. Indication that a lowering of brain Ach occurs as the result of increased release of Ach was directly demonstrated by Mitchell (1963) by the use of the collecting cup technique on the cortical surface. Atropine and hyoscine were shown to increase the release of Ach from the cerebral cortex. Szerb (1964) demonstrated that the application of the cholinesterase inhibitor DFP to the surface of the cortex of non-anaesthetized cats resulted in a low Ach output. Intravenous administration of atropine or the local application of atropine increased Ach output, where the quaternary salt of atropine was 2-4 times less effective. Szerb (1964) also studied the effects of these drugs on the E.E.G. at the same time, and it was concluded that the action of atropine in enhancing the release of Ach occurred directly at the specific site of its application. This effect of the anticholinergics was proven to not be due to the blockade of the vasodilating effects of Ach as the local application of vasodilators or vasoconstrictors did not alter Ach output. The Ach releasing effect of atropine in the brain was also demonstrated by Polak and Meeuws (1966) where they showed increased release of Ach from the ventral surface of the caudate nucleus and

from the cerebral cortex of the rat.

Suggestions as to how the centrally active anticholinergics could reduce the brain Ach were advanced by Giarman and Pepeu in 1964. Here it is stated that they are not likely working by the mechanism of enhancing acetylcholinesterase but rather are producing a block of the central Ach receptors, similar to their action in the periphery, thus physiologically freeing Ach, which is then destroyed by cholinesterase. Any of these cholinergics or anticholinergics which were used to this time to produce this Ach releasing effect in the C.N.S. however, would act as potent cholinergic agonists or antagonists. This could result in the physiological observation of the Ach releasing component of their action not being evident as a biological response.

Anticholinergics may also exert their Ach releasing effect by altering the storage sites of Ach in a manner which reduces the uptake of newly synthesized Ach or by acting in a fashion which increases the release of bound Ach, or perhaps a combination of both these two effects. More recent work carried out on incubated slices of brain tissue presents evidence to indicate that the displacement of bound Ach is one of the mechanisms of their operation. Schubert and Sundwall (1967) studied the effect which various drugs had upon the uptake of Ach by cortex slices of mouse brain. They demonstrated that pentobarbital, pentazol and adrenalin had no effect upon the tissue uptake of exogenous Ach however, hemicholinium-3, eserine, atropine, oxotremorine and morphine all competitively inhibited Ach uptake in the brain. Polak and Meeuws (1966) demonstrated the strong inhibitory effect which eserine had upon the accumulation of Ach in incubated brain slices. Polak (1969) reporting on further studies of

this nature, indicated that hemicholinium-3, O-ethyl S-diethylamino-ethyl ethylphosphonothiolate, eserine, atropine and choline all inhibited the accumulation of Ach in the cortical slices and that the drugs were effective in this regard in the above listed order of potency. It was shown that this uptake of Ach into the cortical slices occurred by active metabolism against a concentration gradient and metabolic inhibitors were effective in arresting the uptake process (Schuberth and Sundwall, 1967).

Polak (1969) investigated the autoradiograms from sections of brain slices in which tritiated Ach had been taken up. He reported that there was no visible preference of the Ach for any certain cell structure but rather it was distributed diffusely throughout the cytoplasm of all the cells. Perhaps this may be taken as some evidence that the Ach taken up is associated with the cytoplasmic labile bound store rather than the vesicular store. This finds support in the findings of Marchbanks (1967) that the vesicles do not take up exogenous Ach. From the work of Polak and Meeuws (1966), Polak (1969) and Schuberth and Sundwall (1967) it appears that drugs inhibiting the uptake of Ach are doing so because of their affinity for anionic binding sites at physiological pH 7.4. Just as drugs may occupy these sites, preventing the binding of Ach in the cell, they are also able to displace Ach from its sites of binding thus resulting in an increase in the release of Ach from the brain.

This literature review contains many references to the binding and release of Ach. Many of them are both directly and indirectly related to the study being reported. The author's opinion as to their pertinence in relation to the results to be reported are found in the discussion.

III. METHODS AND MATERIALS

A. Tremor Induction in Mice

Tremor and/or convulsions were produced by the intracerebral injection of Ach⁽¹⁾, nicotine, oxidized nicotine or one of the nicotine oxidation products in female CF-1 mice weighing between 20-26 g⁽²⁾.

The technique of intracerebral injection in mice was similar to that described by Haley and McCormic (1957). The intracerebral injections were made free hand through the skull administering 1 μ l/g of 0.9 per cent saline solution containing the drug. The pH of all i.c. injection solutions was adjusted to between 6.8 and 7.0 with 1N HCl or NaOH prior to injection. A Hamilton 50 μ l syringe fitted with a 27 gauge needle 2 mm long was used to make the injections. The technique employed is illustrated in Figure 2.

Volumes ranging from 20 to 26 μ l of drug solution were administered to each mouse. This large volume was purposefully used as it represents approximately 5 per cent of the total brain by weight

(1) Acetylcholine iodide, J.T. Baker Chemical Co.

(2) The tremorogenic effect of intracerebrally injected Ach and the effect of HC-3 treatment on nicotine-N-oxide tremor was tested using an inbred strain of mice developed at the Defence Research Establishment Suffield. Here male mice weighing between 23-36 g were used.

Figure 2

Free Hand Intracerebral Injection Into the Mouse.



and would be much more than the cerebral spinal fluid volume in the cerebral region according to Haley and McCormic (1957). The distribution of the injected solution through the brain was checked by injecting the same volume of saline containing 10 per cent India ink as a marker. The brains from 20 mice thus treated were removed 3 minutes after injection and fixed in 10 per cent formaldehyde. Gross sections 1 mm thick, from these fixed brains were prepared and the distribution of dye marker particles observed. It was found that the solution injected "flooded" the entire brain, with marker particles being found in the lateral ventricles, the aqueduct and the third and fourth ventricles. This was similar to the results reported by Cashing and Heading (1968) who also used a relatively large volume for injection ($20 \mu\text{l}/\text{mouse}$) to "flood" the total brain with drug solution. The technique of intracerebral injection in mice has been utilized by other workers (Gokhale and Gulati, 1962; Decsi, Varszegi and Mehes, 1963; Clark, Vivonia and Baxter, 1968; and Takagi and Iwasawa, 1968). In this study however, similar to that previously reported by Madill, Stewart and Savoie (1968), it is to be emphasized that the technique of intracerebral injection performed under the conditions of the experiment, only represents a means of putting drugs into the whole brain for observation of their immediate central effects. This may be likened to the bathing of an isolated organ in a tissue bath.

All intracerebral drug injections were compared to the effect which control injections of the same volume of saline had upon mice. Any effect which this control injection had lasted for not more than 15 seconds and was usually manifested by a "stunning" effect, presumably because of the resulting sudden increase in intra-

cerebral pressure. After recovering from this initial effect all control mice appeared normal.

When the tremorogenics, Ach, nicotine, oxidized nicotine or any of the nicotine oxidation products were injected; their effect was not scored until 30 seconds after the time of injection. The treated mice were observed for 10 minutes after injection as preliminary experimentation had revealed that the tremorogenic effect of any of the drugs tested was usually maximal within 3 minutes after injection and had subsided within 5 to 7 minutes after the injection. The onset of tremor following treatment was scored subjectively by close observation of the mice in individual lucite boxes with the following scoring system being used:

- + tremor response just perceptible but not persistent;
- ++ tremor response persisting for 30 seconds or more after the beginning of scoring;
- +++ tremor response accompanied by hyperactivity and clonic convulsions;
- ++++ immediate clonic convulsions often termination in tonic extensor convulsions and death.

Only mice which showed a score of ++ or greater were recorded as positive respondents to drug treatment. In this fashion a quantal response of mice to drug treatment was recorded. Due to the quantal system of symptom assessment no distinction was made between tremor or convulsions. A much greater number of mice treated would have been required to quantitate this difference. Ten mice were tested at each dose of tremorogenic drug used, in most cases; with four to twelve doses being employed to establish a dose-response line for each compound. The dose response lines and ED₅₀'s for response were

calculated by the method of probit analysis (Finney, 1947). When statistically similar slopes of these lines were proven, relative potencies between the ED_{50} s were calculated. All ED_{50} s for Ach, nicotine, oxidized nicotine or oxidation products of nicotine are reported as mg/kg of equivalent base of the compound.

A sample of pure alkaloidal nicotine was oxidized using activated manganese dioxide. The method used by the author was after that described by Hirst (1968). The procedure consisted of adding 10 g of activated MnO_2 to 10 ml of alkaloidal nicotine in 200 ml of benzene. The mixture was stirred at room temperature for 18 hours. The solution was then filtered and the benzene removed under vacuum at $38^\circ C$. The resulting material was dark brown in color.

Nicotine-1'-oxide was synthesized by the author after the method described by Taylor and Boyer (1959). This sample was characterized by conversion to its dipicrate salt which showed a melting point of $164-167^\circ C$, and C, H, N analysis of 41.11 per cent, 3.40 per cent and 17.40 per cent respectively, compared to 41.5 per cent, 3.2 per cent and 17.6 per cent theoretical.

Of the other auto-oxidation products reported to be present in air oxidized nicotine by Wada et al (1959) nicotinic acid⁽¹⁾, β nicotine⁽²⁾ and cotinine⁽³⁾ were tested for their tremorogenic potency in mice before and after DFP.

(1) Aldrich Chemical Company

(2) Aldrich Chemical Company

(3) Synthesized by Dr. S.K. Ghandi, Department of Pharmacology, University of Western Ontario.

Thin layer chromatography was carried out on the samples of nicotine and oxidized nicotine used in this study. Commercial silica gel plates⁽¹⁾ 250 microns thick using the solvent systems of; 1:1:4, 0.5 N NH_4OH , EtOH and n-butanol and 8:10:1:1, acetone, benzene, EtOH and conc. NH_4OH . After development, the spots were visualized on the plate by spraying it with 1 per cent para-amino benzoic acid in absolute EtOH and exposing it to CNBr vapour, using the Koning color reaction for the indication of tertiary pyridine derivatives as described by Kodicek and Reddi (1951).

B. Drugs Tested for Their Effects Upon Ach, Nicotine and Oxidized Nicotine Induced Tremor

The anticholinesterase agents DFP and Soman were administered by intravenous injection into the lateral tail vein of the mouse. Solutions of these agents were prepared fresh in saline on each day they were to be used and administered to the mice at a volume of 0.005 ml/g. The lethal potency of the anticholinesterases were determined in mice similarly injected and observed for 24 hours. The LD_{50} of DFP was found to vary between 3.50 and 4.0 mg/kg depending upon the particular sample being used, while the LD_{50} for the sample of Soman available was found to be 0.08 mg/kg. Dose response lines and the 95 per cent confidence intervals calculated by the method of probit analysis (Finney, 1947), for these two agents are found in Appendices I and II.

The LD_{50} dose of DFP was determined accurately and frequently by intravenous injection in mice. When the LD_{50} of a particular sample was found to have increased by 10 per cent, a fresh sample of agent was tested and used.

⁽¹⁾Silicar TLC-7G. E.H. Sargent and Company.

The dose of anticholinesterase chosen to pretreat the mice prior to testing them with a tremorogenic was equal to 1/2 of the intravenous LD₅₀. This dose was chosen as it represents the threshold sub-tremorogenic dose of DFP, yet was sufficient to significantly depress brain acetylcholinesterase. This is in agreement with the findings of Frawley, Hagen and Fitzhugh (1952) where it was demonstrated that animals with only 8 per cent of their normal brain acetylcholinesterase activity remaining after treatment with DFP exhibited only minimal symptoms of anticholinesterase poisoning. One half the LD₅₀ dose of soman also did not produce tremor as a symptom of anticholinesterase poisoning.

The drugs atropine⁽¹⁾ and mecamlamine⁽²⁾ were administered by intraperitoneal injection in saline at a volume of 0.005 ml/g of mouse. This treatment was given 30 minutes before the animals were challenged by intracerebral injection with a tremorogenic. When mice were treated with hemicholinium-3⁽³⁾, it was administered one hour prior to the tremorogen by intracerebral injection. Thus, such mice received two intracerebral injections, one through each side of the skull, one hour apart.

C. Brain Acetylcholinesterase Measurement in Mice after DFP

Brain acetylcholinesterase levels were determined in mice 30 minutes after receiving varying doses of DFP given by i.v. injection. This time period is believed to be more than adequate for penetration of the DFP into the brain to produce cholinesterase inhibition,

(1) atropine sulphate - The British Drug Houses Limited.

(2) mecamlamine hydrochloride - Merck Sharp and Dohme.

(3) hemicholinium-3 - Aldrich Chemical Company.

according to the work of Mazur and Bodansky (1946). It was also the same time period used by Holmstedt, Harkonen, Lundgren and Sundwall (1967) who studied brain cholinesterase depression in rats after i.p. injections of 2(diethoxyphospinylthio)-ethyltrimethylammonium hydrogen oxalate.

The brain acetylcholinesterase levels were determined using the method based upon automatic titration of the liberated acid following hydrolysis of a known amount of substrate by a sample of brain tissue acetylcholinesterase. This method is similar to that described by Jensen-Holm, Lausen, Milthers and Møller (1959) and makes use of the automatic titrator and titrigrath by Radiometer.

Thirty minutes after DFP treatment, the mouse was decapitated, the brain removed, blotted, and weighed and then immersed in a homogenization vessel containing 50 ml/g tissue wet weight, of ice cold saline. It was then homogenized at 12,000 RPM for 3 minutes using a Sorval-Omni Mixer. One ml of this homogenate was added to 7 ml of saline in a 10 ml reaction vessel of the titrimeter. After the temperature was allowed to equilibrate at 37° C for 5 minutes, 1.0 ml of saline containing 50 mg acetyl β methylcholine chloride was added to the reaction vessel and the recording commenced. The change in pH occurring as the result of enzyme hydrolysis of the substrate was counteracted by automatic titration with 0.005N NaOH. This reaction was allowed to proceed for a minimum of fifteen minutes with the amount of NaOH added being recorded on the titrigrath. The blank rates of NaOH addition occurring when acetyl β methylcholine chloride was added to saline in the absence of brain homogenate, were subtracted from the rates of addition required when tissue from normal mice and mice given various doses of DFP were tested. Seven mice were tested

at each dose of DFP used as well as seven mice tested to establish the normal brain acetylcholinesterase level in untreated mice. Each mouse homogenate was tested three times and a mean activity calculated from the three readings, replicated seven times, was obtained.

Testing was carried out to determine if the brain homogenization procedure produced an effect of causing readings in brain acetylcholinesterase activity which were not indicative of the levels occurring in vivo (Heath, 1961). Such an anomaly had been suggested as possibly occurring due to homogenization liberating normally protected acetylcholinesterase or that the presence of any active DFP in the brain may produce further inhibition in vitro during the time of brain homogenization. A check was carried out to determine if this was taking place using the system of diluting the homogenate from the brains of mice which had been given DFP with a brain homogenate obtained from normal mice which received no pretreatment.

The procedure employed using this technique was as follows: A mouse was treated with 1.3 mg/kg of DFP i.v. and after 30 minutes the brain removed and homogenized using the same procedure described for the measurement of DFP brain acetylcholinesterase depression. The acetylcholinesterase activity of this homogenate was then measured. Equal volumes of this sample were then combined with a sample of brain homogenate obtained from a normal untreated mouse and the acetylcholinesterase activity of this mixture measured. This procedure was replicated four times.

D. Brain Acetylcholine Measurements

The method used for Ach extraction and separation of "free" and "bound" Ach was similar to that described by Crossland and Slater (1968). In this instance "free" Ach is the portion removed by homogenization, at 1200 RPM for 5 minutes using a Lourd's Model MM-1A multimixer, in 5 ml/g of tissue wet weight ice cold saline containing eserine sulphate, 15 mg/l and cupric chloride, 17 mg/l. The homogenate then was centrifuged at 2° C at 17,000 x G. for 10 minutes and the supernatant removed, adjusted to pH 4.0, and stored frozen until the time of assessment. The "bound" Ach was then extracted from the residue by re-homogenization at 12,000 RPM for 5 minutes in 5.0 ml/g ice cold absolute EtOH and 0.2 per cent glacial acetic acid. The samples were centrifuged as before and the supernatant removed. The tissue was re-homogenized two more times with 2.5 ml/g of ice cold 75 per cent EtOH and 1.5 per cent glacial acetic acid, using the same speed of homogenization each time for a period of 3 minutes and the same procedure of centrifugation. The supernatants from each extraction of "bound" Ach were combined. Five ml of water were then added to each sample and the volume reduced to approximately 2.0 ml by evaporation under a stream of air while the samples were maintained at 40° C. The pH of each sample was then adjusted to 4.0 and it was stored as frozen until the time of analysis.

Four mouse brains were pooled to make each sample to be extracted. In all cases where i.c. injections were given, the mice were decapitated three minutes after the injection and the brains were removed and immersed in the ice cold saline solution, containing eserine and cupric chloride, within 45 seconds after decapitation. Each brain was divided into several smaller pieces with a spatula upon

being immersed in the saline solution. Each treatment extraction was replicated eight times, thus Ach readings to be subsequently reported are the means of eight readings, unless otherwise noted.

The assays for Ach were performed using the frog rectus abdominis muscle sensitized with eserine $1 \times 10^{-5}M$, after the method described by MacIntosh and Perry (1950). After thawing and equilibration to room temperature, the pH of each brain sample to be assayed was adjusted to 6.8 while stirring with 0.5 N NaOH just prior to testing.

A portion of each sample was prepared for a control blank by alkaline hydrolysis (pH adjusted to 11.0 with 0.5 N NaOH, stirred at room temperature for 15 minutes, then returned to pH 7.0 with 0.5 N HCl for assay). Equal dilutions of brain extract with Ringer's solution were made for both the control samples containing known amounts of Ach, and the unknown samples which were assayed. Effort was made to prepare the control samples with known amounts of Ach to produce muscle contractions just less than and just greater than those produced by the brain samples being assayed. The Ach content of the sample was then estimated by interpolation on the basis of the height of the recorded muscle contractions of the two control samples; one tested before the unknown sample and one tested after. In all cases the dilutions of the brain extracts employed were sufficiently low as to produce no muscle contraction by the control samples containing no added Ach. This technique is similar to that described by Feldberg (1945a), to take into account the presence of unknown substances in the brain extract which may sensitize the muscle to Ach. The authenticity of Ach in the extract being measured was checked by the

removal of the activity of the extract by the alkaline hydrolysis procedure just described, as well as the equal diminution of the response of the muscle to the Ach standards and the extracts following the addition of d-tubocurarine or atropine to the muscle bath.

It is well known that the frog rectus abdominis muscle is very sensitive to the effects of nicotine and its response to nicotine-N-oxide is also reported (Nicotine-1'-oxide having 1/20 the activity of nicotine according to Arutyunyan and Mashkovski, 1961). Theoretical maximum concentrations in the brain extracts of these two substances were calculated at the two highest doses administered, i.e. 20.0 mg/kg for nicotine-N-oxide and 0.1 mg/kg for nicotine. Upon adding these amounts to control brain extracts which had been subjected to alkaline hydrolysis, they failed to elicit a response in the muscle. The threshold for activity of these two substances under the conditions of the assay were found to be 1×10^{-6} M for nicotine and 4×10^{-4} M for nicotine-N-oxide by bio assessment on the frog rectus abdominis muscle; whereas the maximum concentrations possible in the mouse brain extracts after the two highest doses administered would be 1.25×10^{-7} M for nicotine and 2×10^{-5} M for nicotine-N-oxide, even if one were to assume there was no translocation of these compounds to other parts of the body during the three minutes time period between injection and decapitation. In addition, active concentrations of these two nicotine substances were added to mouse brain extracts which were then subjected to alkaline hydrolysis at pH 11.0 for 15 minutes at room temperature and then returned to pH 7.0. The biological activity of each of these compounds was not affected by this treatment. During the course of the bioassay, control

blank samples were frequently tested for activity when added to the bath in the same concentration as the unknown extracts to be tested, and it was always found that they were without any activity on this organ preparation. If the sub-active concentrations of nicotine or nicotine-N-oxide present in the brain extracts in any way affected the sensitivity of the frog rectus abdominis muscle to Ach; the procedure employed would presumably take this into account. Thus the brain extracts containing the standard amounts of Ach and those being assayed for their unknown amount of Ach would both be affected in a similar manner, rendering the assay uniform.

IV. RESULTS

A. The Effect of DFP Pretreatment Upon Mouse Brain Acetylcholinesterase

The results obtained from the measurement of brain acetylcholinesterase following the treatment with DFP are illustrated in Figure 3, and are expressed as per cent of normal brain acetylcholinesterase activity with the corresponding S.E. of each mean reading indicated. These standard errors were calculated on the transformed percentage data. Here it is shown that the $1/2$ LD₅₀ dose of DFP used throughout this study depressed mouse brain acetylcholinesterase to a value of 9.4 ± 0.17 per cent of normal.

The results obtained from the procedure employed to check if brain homogenization was producing incorrect brain acetylcholinesterase readings were as follows: The mean acetylcholinesterase activity from four experiments indicated that the mouse brain acetylcholinesterase was reduced to 31.5 per cent of normal 30 minutes after being given 1.3 mg/kg of DFP. When these samples were diluted 1:1 with mouse brain homogenate from normal mice, the acetylcholinesterase level was found to be 64.5 per cent of normal. These levels of brain acetylcholinesterase depression produced by DFP correspond to those which lie upon the linear portion of the DFP brain acetylcholinesterase depression curve reported in Figure 3, and represent an almost absolute 2-fold increase in activity due to the 1:1 dilution employed. This doubling of activity is well within the experimental error of the assay.

It thus may be assumed that the brain acetylcholinesterase activities following DFP treatment reported in Figure 3 are indicative of the activity of this enzyme occurring in mouse brain tissue tested and that the procedure of brain homogenization employed did not enhance the depression recorded. This acetylcholinesterase depression is attributed to acetylcholinesterase enzyme inhibition by DFP.

Even though this high level of acetylcholinesterase depression was obtained with this dose of DFP, symptoms of anticholinesterase poisoning in these animals were slight with only some individuals showing salivation and urination.

B. The Tremorogenic Effect of Intracerebrally Injected Acetylcholine
In Mice

The results obtained from the intracerebral injection of Ach in producing tremor and/or convulsions in mice are illustrated in Figure 4. Here the ED_{50} s for tremor and/or convulsions of Ach administered alone or 30 minutes following $1/2 LD_{50}$ of DFP; atropine 3.0 mg/kg; DFP plus atropine 3.0 mg/kg; and DFP plus mecamylamine 1.7 mg/kg are shown with their corresponding 95 per cent confidence limits. The data from which these dose response results were calculated are tabulated in Appendix III. The calculated dose response lines were proven to be parallel and relative potencies between the resulting ED_{50} s were calculated. As was expected DFP pretreatment produced a highly significant potentiation in the tremorogenic effect of the Ach administered ($93X$, $p < 0.001$). The administration of atropine significantly protected the mice against the tremorogenic effects of the Ach injection. The level of this protection was limited by the lethal effects of high doses of Ach encountered

FIGURE 3

Whole Brain Acetylcholinesterase Activity in Female CF-1 Mice
30 Minutes After Intravenous DFP; Expressed as the Per Cent
of Normal Acetylcholinesterase Activity, with the Corresponding
Standard Errors of Each Mean Reading, ($n = 7$).

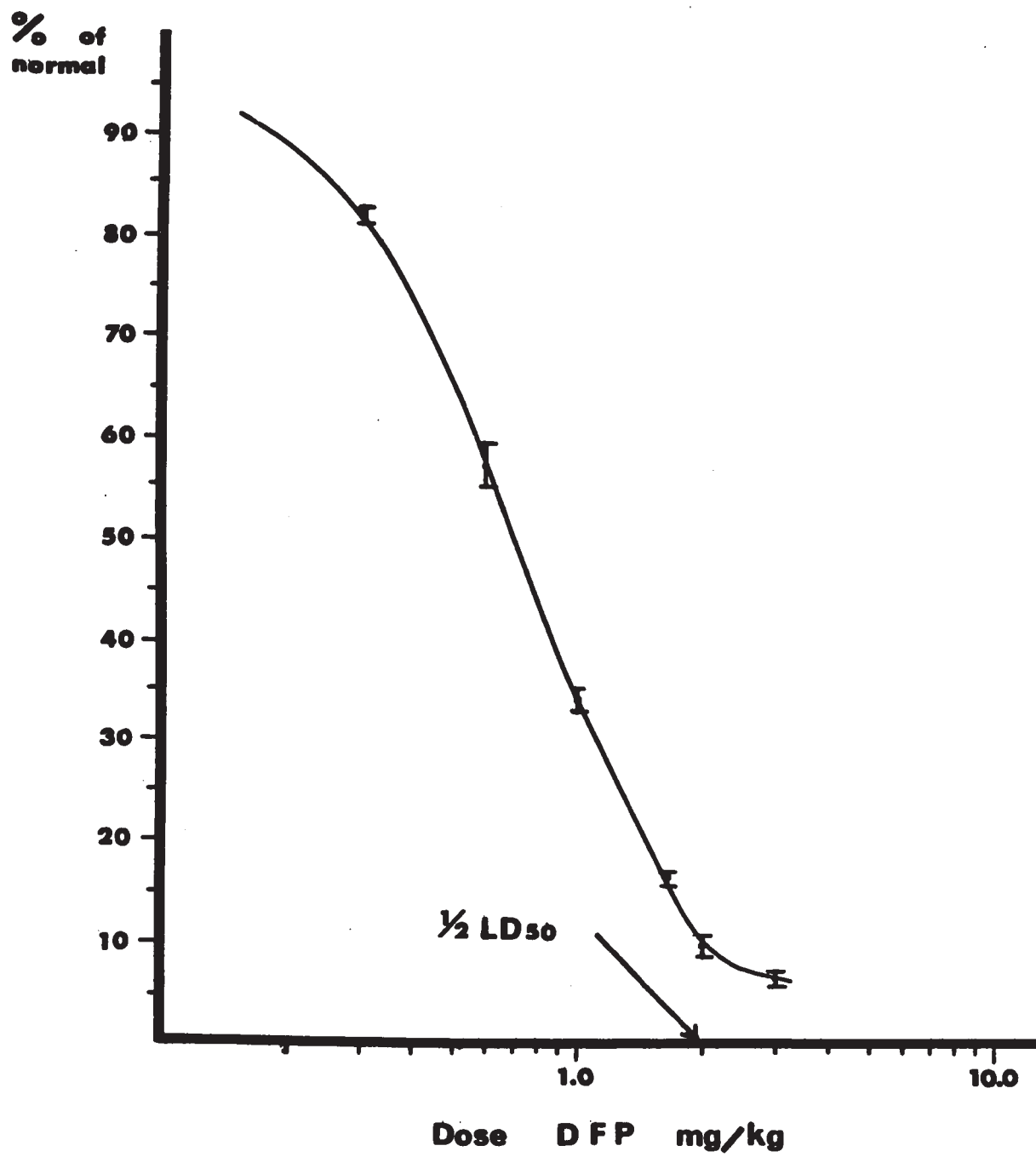
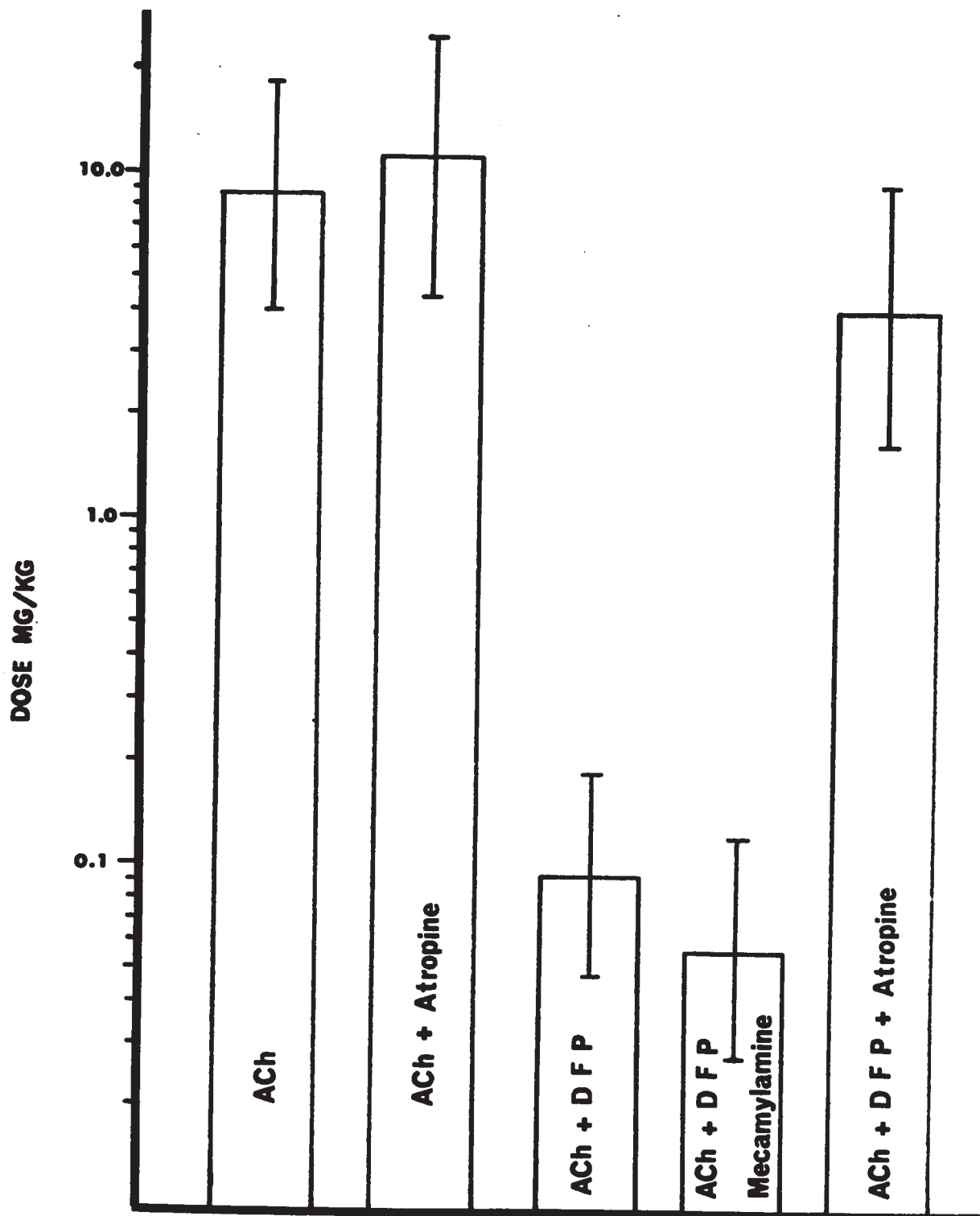


FIGURE 4

ED₅₀s and Their Corresponding 95 per cent Confidence Limits
 for Tremor and/or Convulsions Following the i.c.
 Administration of Acetylcholine to Mice, Alone and 30 Minutes
 Following Treatment with Atropine 3.0 mg/kg;
 DFP; DFP plus Mecamylamine 1.7 mg/kg and DFP plus Atropine 3.0 mg/kg.

TREMOR ED₅₀



The LD_{50} for Ach given after 3.0 mg/kg of atropine was 73.0 mg/kg (32.0 - 131.0), which was statistically proven to be greater than the ED_{50} for tremor and/or convulsions following this same treatment ($p < 0.01$) but only by a factor of 5X, and the slopes of the dose response curves for tremor and death were parallel. This is indicated in the data presented in Appendix III. Atropine administered at the same time as the DFP significantly blocked the effect of the Ach injection ($p < 0.001$). The ED_{50} for this treatment was not significantly different from that of Ach given alone. The most significant finding in these drug effects upon the tremorogenic potency of i.c. administered Ach was that mecamlamine offered no protection at all against the effect of Ach given to DFP treated mice.

C. The Tremorogenic Effect of Intracerebrally Injected Nicotine in Mice

The results obtained from the tremorogenic response of mice following intracerebrally injected nicotine when administered alone or thirty minutes after DFP; DFP and 3.0 mg/kg atropine; mecamlamine 1.7 mg/kg; and DFP plus mecamlamine 1.7 mg/kg, yielded dose response lines which were parallel. The ED_{50} s for tremor and/or convulsions and the relative potencies between them were calculated by the method of Probit Analysis. These ED_{50} s with their 95 per cent confidence limits are illustrated in Figure 5 with the data which yielded these results appearing in Appendix IV. Relative potency analysis indicated that there was no significant effect upon the nicotine tremor produced by pretreating the mice with DFP or DFP and atropine. The nicotine tremor was significantly blocked by pretreating the animals with 1.7 mg/kg of mecamlamine or this dose of

mecamylamine given at the same time as DFP. The protection afforded in these two instances was respectively found to be equal to 72X and 111X the ED_{50} of nicotine given alone. No significant difference was shown to be produced by the DFP treatment upon the nicotine blocking effect of mecamylamine.

D. The Tremorogenic Effect of Intracerebrally Injected Auto-Oxidized Nicotine in Mice

When a sample of naturally oxidized nicotine was tested in a similar manner to nicotine bitartrate, the dose response lines for tremor and/or convulsions were also parallel. This auto-oxidized sample of alkaloidal nicotine was dark in color as it had been stored at room temperature for an excess of two years. The ED_{50} s for tremor following the use of this sample in combination with the various drug treatments previously described, are illustrated in Figure 6. The data yielding these results are found in Appendix V. Relative potency analysis between the resulting ED_{50} s in this instance indicated that DFP pretreatment produced a significant, 6X, potentiation in the nicotine tremor. Atropine given at the same time as the DFP appeared to partially remove this potentiating effect as it was then found to be only 3.8X. This difference between the ED_{50} s following DFP treatment and DFP atropine treatment is not significant ($p > 0.05$).

As was noted in the instance of the use of pure nicotine, highly significant levels of protection against the oxidized nicotine tremor were shown by treating the mice with 1.7 mg/kg of mecamylamine or mecamylamine given with DFP. This protection was 21X the ED_{50} of oxidized nicotine when mecamylamine was given and 13X the ED_{50} of oxidized nicotine when mecamylamine and DFP were given. In this

FIGURE 5

ED₅₀s and Their Corresponding 95 per cent Confidence Limits for Tremor and/or Convulsions Following the i.c. Administration of Nicotine to Mice, Alone and 30 Minutes Following Treatment with DFP; DFP plus Atropine 3.0 mg/kg; Mecamylamine 1.7 mg/kg and DFP plus Mecamylamine 1.7 mg/kg.

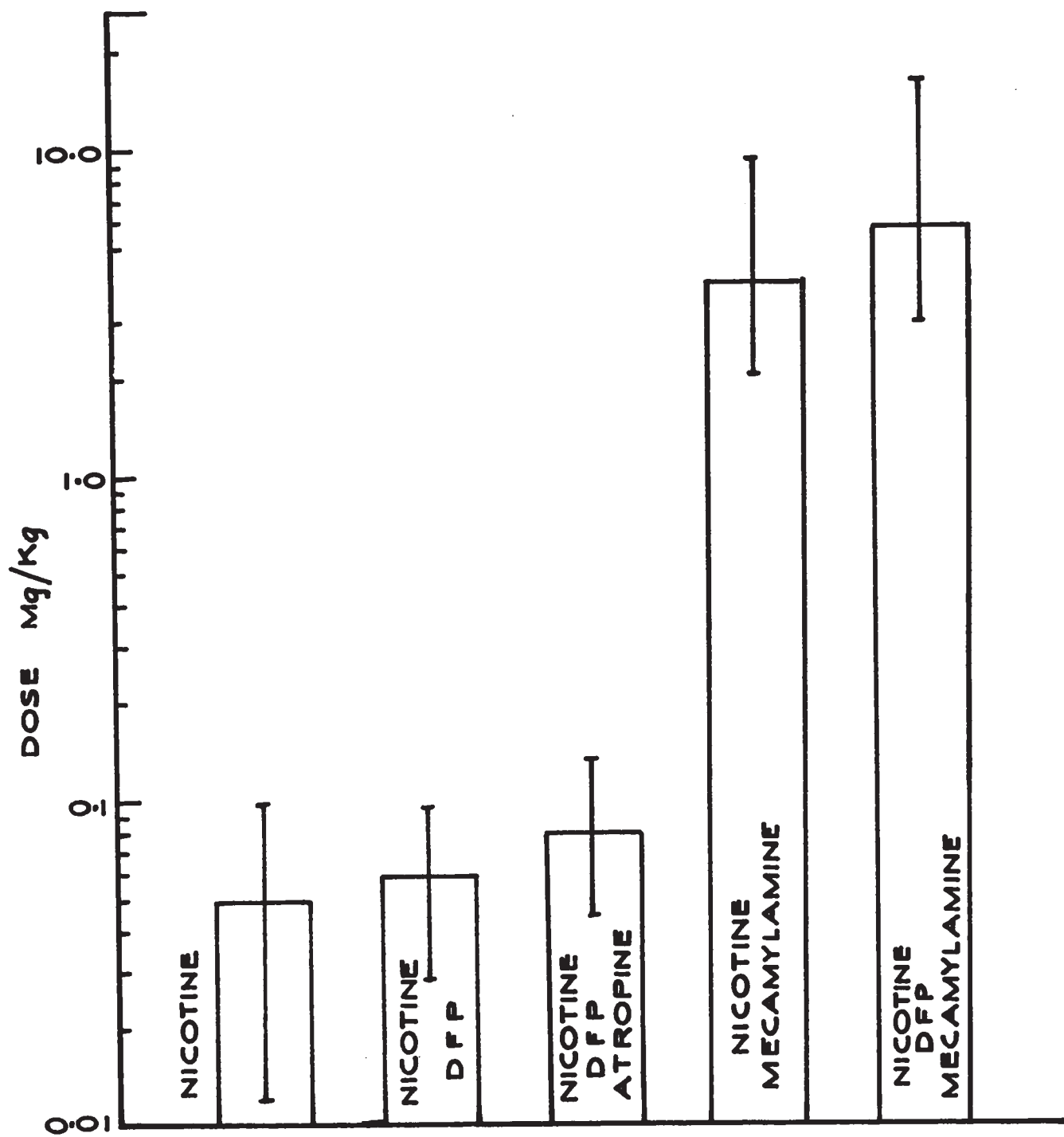
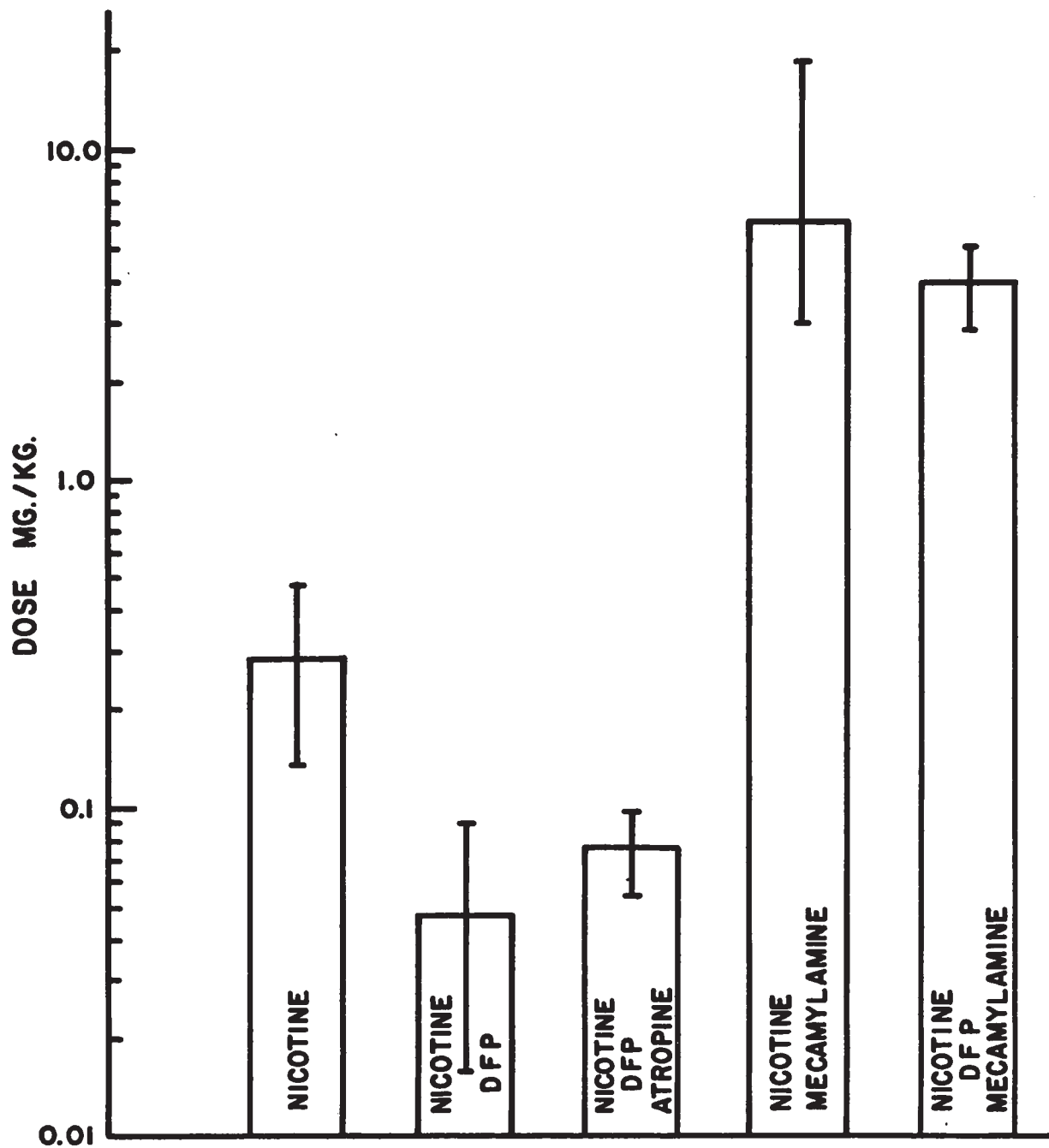


FIGURE 6

ED_{50} s and Their Corresponding 95 per cent Confidence Limits for Tremor and/or Convulsions Following the i.c. Administration of Auto-oxidized Nicotine to Mice, Alone and 30 Minutes Following Treatment with DFP; DFP plus Atropine 3.0 mg/kg; Mecamylamine 1.7 mg/kg and DFP plus Mecamylamine 1.7 mg/kg.



instance the treatment with the DFP appears to be partially removing the protection effected by the mecamlamine, however, this difference is not significant ($p > 0.05$).

E. The Tremorogenic Effect of Intracerebrally Injected MnO_2
Oxidized Nicotine in Mice

The dark brown MnO_2 oxidized nicotine was tested for its tremorogenic effects in mice both alone and with the previously used combinations of DFP, atropine and mecamlamine.

In this case the resulting dose response lines for tremor and/or convulsions were not parallel for in all cases where DFP was given prior to the nicotine there was a significant flattening of the dose response lines. This is illustrated in Figure 7. The ED_{50} s with their 95 per cent confidence limits obtained from these calculated dose response lines are shown in Figure 8. The data yielding the results reported in Figures 7 and 8 appear in Appendix VI. Here a profound reduction in the ED_{50} of oxidized nicotine following DFP pretreatment is noted. The ED_{50} of the oxidized nicotine alone was found to be $199 \mu\text{g/kg}$ and only $19 \mu\text{g/kg}$ after DFP. Although parametric methods of statistical comparison between these two dose response lines were not possible, inspection indicated that the effects of the DFP pretreatment potentiated the tremorogenic effect of the oxidized nicotine 10 times. A similar situation occurred to that found with the naturally oxidized sample of nicotine in that atropine partially removed this potentiating effect. In the instance of mice pretreated with atropine and DFP, the ED_{50} for tremor and/or convulsions was $67 \mu\text{g/kg}$ for the MnO_2 oxidized nicotine.

FIGURE 7

Dose Response Lines for Tremor and/or Convulsions Following the i.c. Administration of MnO_2 Oxidized Nicotine to Mice, Alone and 30 Minutes After DFP; DFP plus Atropine 3.0 mg/kg; Mecamylamine 1.7 mg/kg and DFP plus Mecamylamine 1.7 mg/kg.

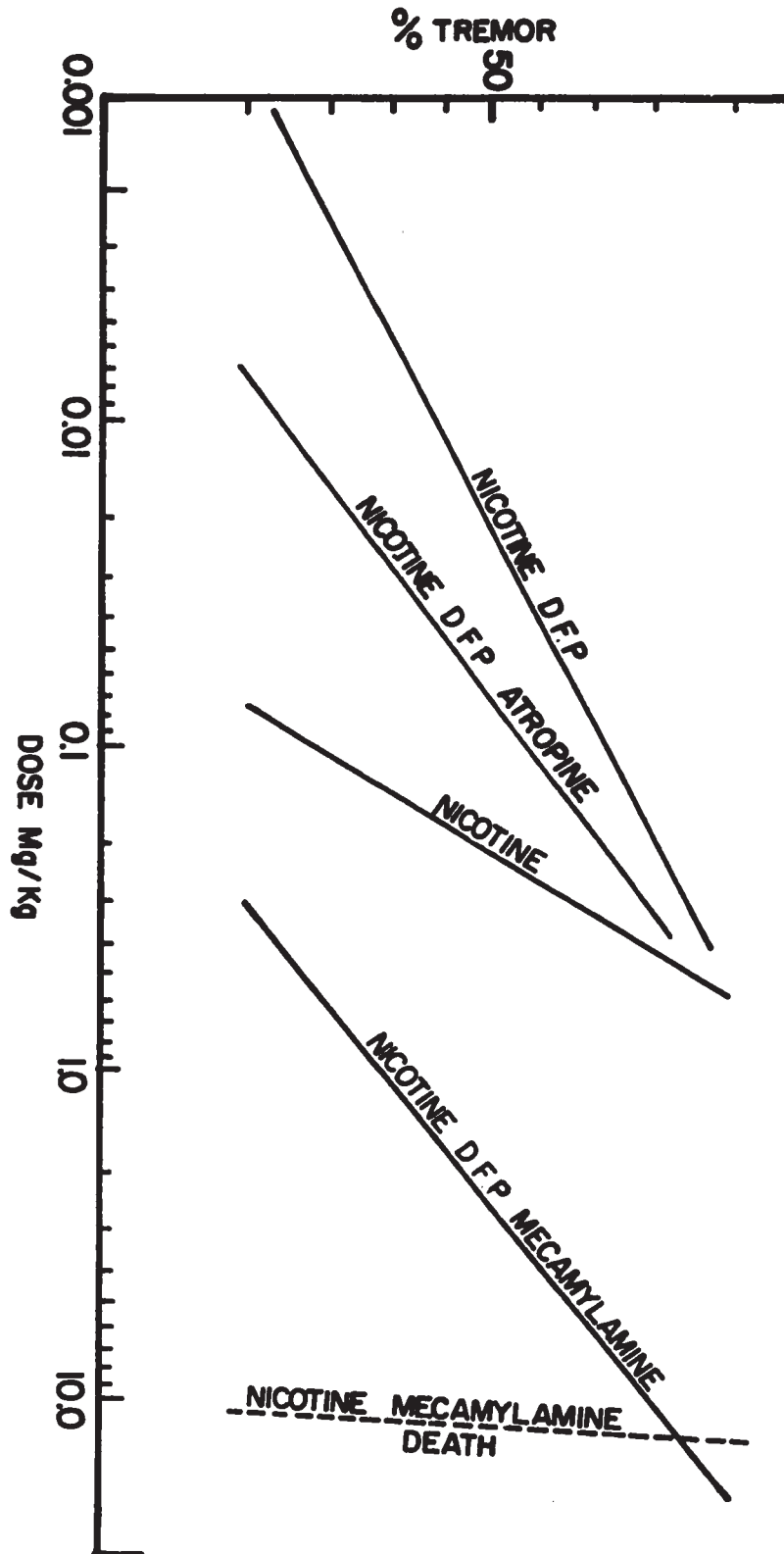
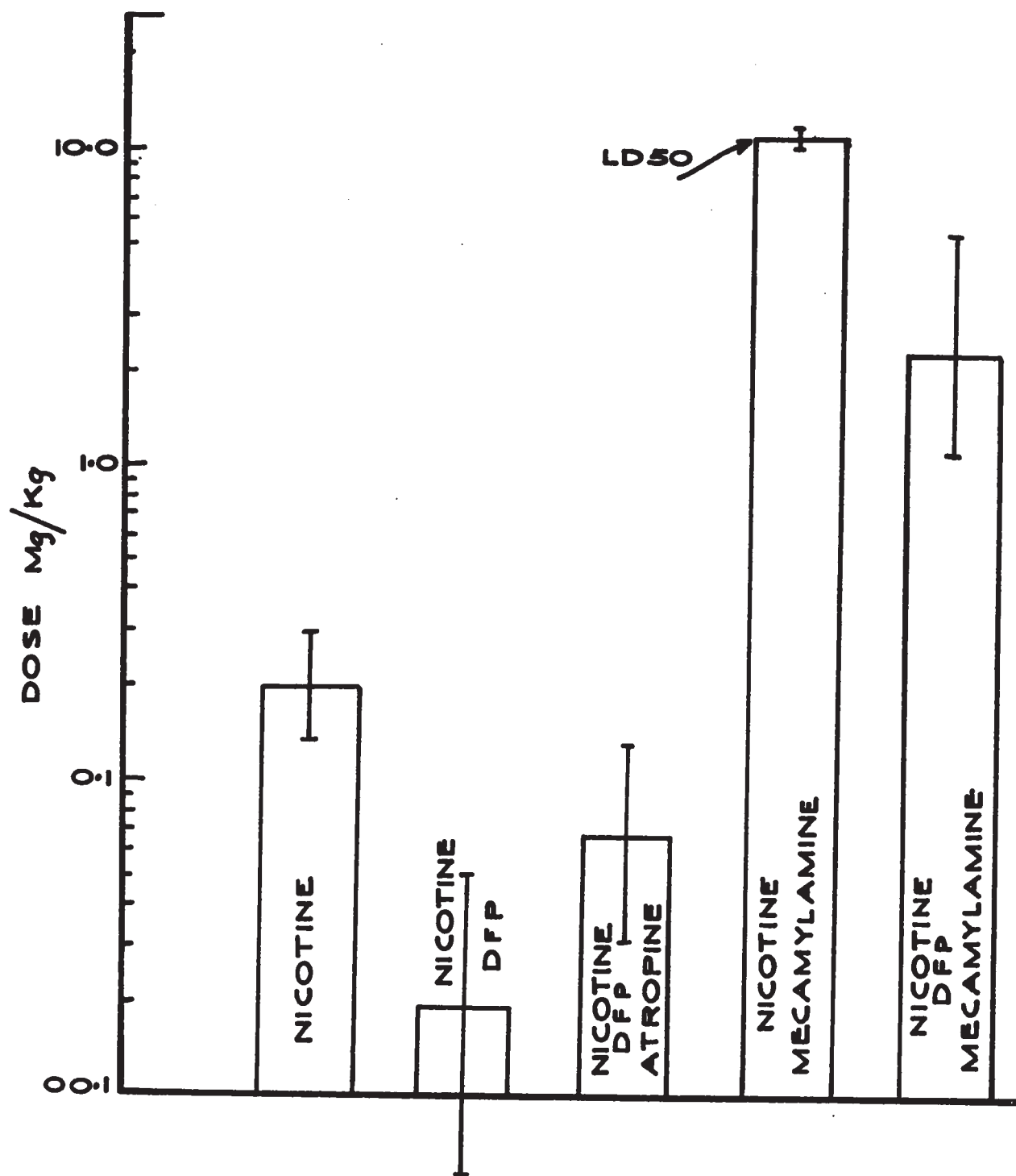


FIGURE 8

ED₅₀s and Their Corresponding 95 per cent Confidence Limits for Tremor and/or Convulsions Following the i.c. Administration of MnO₂ Oxidized Nicotine, Alone and 30 Minutes Following Treatment with DFP; DFP plus Atropine 3.0 mg/kg; Mecamylamine 1.7 mg/kg and DFP plus Mecamylamine 1.7 mg/kg.



It is indicated in both Figures 7 and 8 that mecamlamine was effective in blocking the tremor or convulsions produced by the MnO_2 oxidized nicotine up to the dose which caused death. In this instance, DFP pretreatment reduced the blocking effect of the mecamlamine pretreatment. The LD_{50} of the oxidized sample given after mecamlamine was 11.3 mg/kg and the ED_{50} for tremor and/or convulsions when mecamlamine and DFP had been previously given was 2.4 mg/kg.

The effect of using the anticholinesterase inhibitor Soman prior to the administration of an intracerebral injection of nicotine and MnO_2 oxidized nicotine was investigated. In this case, a qualitative similar shift to the left and flattening of the dose response line was found, similar to that observed when DFP was used as the cholinesterase inhibitor. This occurred with MnO_2 oxidized nicotine while no effect of the Soman was observed upon the tremorogenic dose response of nicotine (Figure 9). The ED_{50} s with their 95 per cent confidence limits derived from these dose response lines in Figure 9 are shown in Figure 10. The data from which these figures were derived are found in Appendix VII. In this case it was also found that parametric statistical means of comparison of the ED_{50} s of the MnO_2 oxidized samples of nicotine following the various drug treatments were not possible but inspection indicated that pretreatment with Soman potentiated the tremorogenic effect of this oxidized nicotine 4 X.

F. Nicotine Oxides and Their Tremorogenic Effect in Mice

The results obtained from the thin layer chromatograms are illustrated in Figures 11 and 12. Here it is evident that there are as many as seven different Koning positive spots present in the sample of nicotine which was oxidized by MnO_2 . The most predominant of these spots had R_f values and color reactions corresponding to those of cotinine, β nicotine and nicotine itself. The sample of β nicotine showed a primary spot giving the red Koning color reaction for this product (Tso and Jeffrey, 1953), however it also showed a smaller spot which had a mobility and color reaction corresponding to that of nicotine. The sample of cotinine used produced only a single spot under both chromatographic separation systems. The chromatograms revealed the presence of small amounts of two other substances other than the primary spot believed to be nicotine-1'-oxide. One of these spots gave a similar R_f value and Koning color reaction to that of cotinine while the other remained unidentified throughout the course of the remainder of the biological testing. Taylor and Boyer (1959) reported on the occurrence of three possible nicotine-N-oxides; these being nicotine-1-oxide, nicotine-1, 1'-dioxide and nicotine-1'-oxide which are shown in Figure 13. It is for this reason that the sample of nicotine-1'-oxide prepared is referred to as nicotine-N-oxide throughout the course of the biological testing. This sample was stored under refrigeration at 2°C for the duration of the investigation. At the end of the period of biological testing a sample of the nicotine-N-oxide was analyzed by Nuclear

FIGURE 9:

Dose Response Lines for Tremor and/or Convulsions
in Mice After i.c. Injections of Nicotine and MnO_2
Oxidized Nicotine, Both Alone and 30 Minutes After
 $\frac{1}{2}$ LD₅₀ of i.v. Soman.

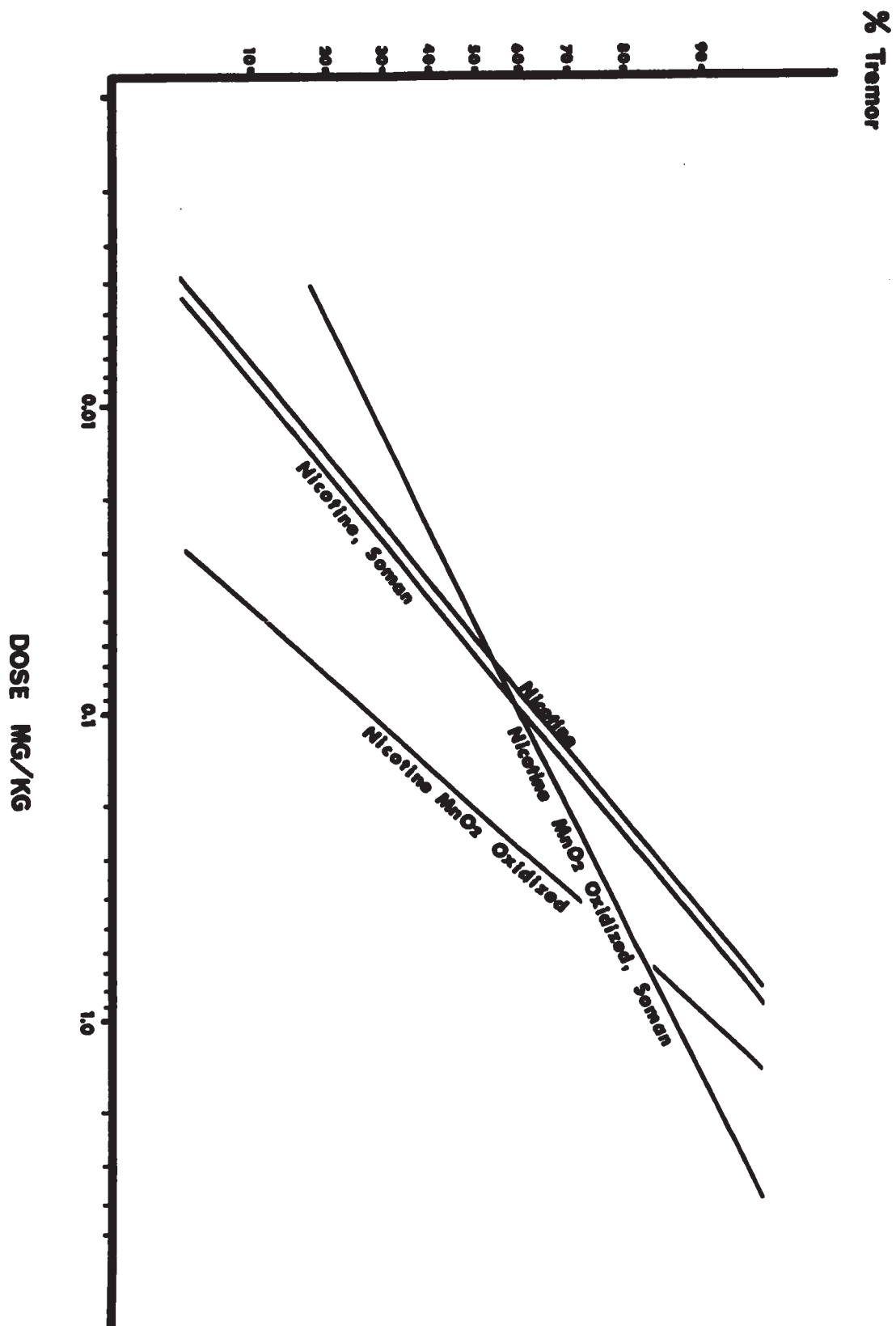


FIGURE 10

ED₅₀s and Their Corresponding 95 Per Cent Confidence Limits
for Tremor and/or Convulsions Following the i.c. Administration
of Nicotine and MnO₂ Oxidized Nicotine, Alone and 30 Minutes
Following Treatment with 1/2 LD₅₀ of i.v. Soman.

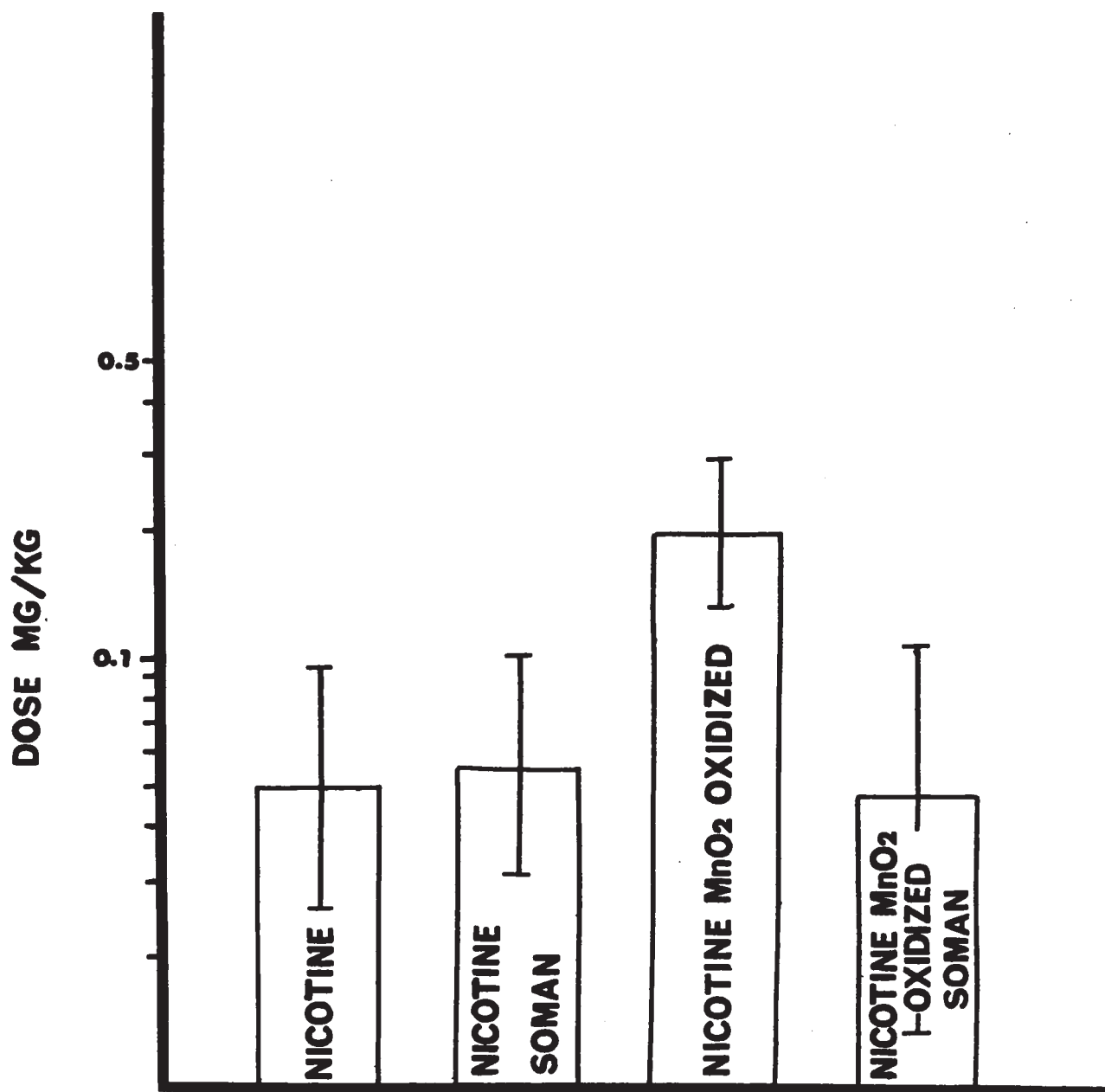
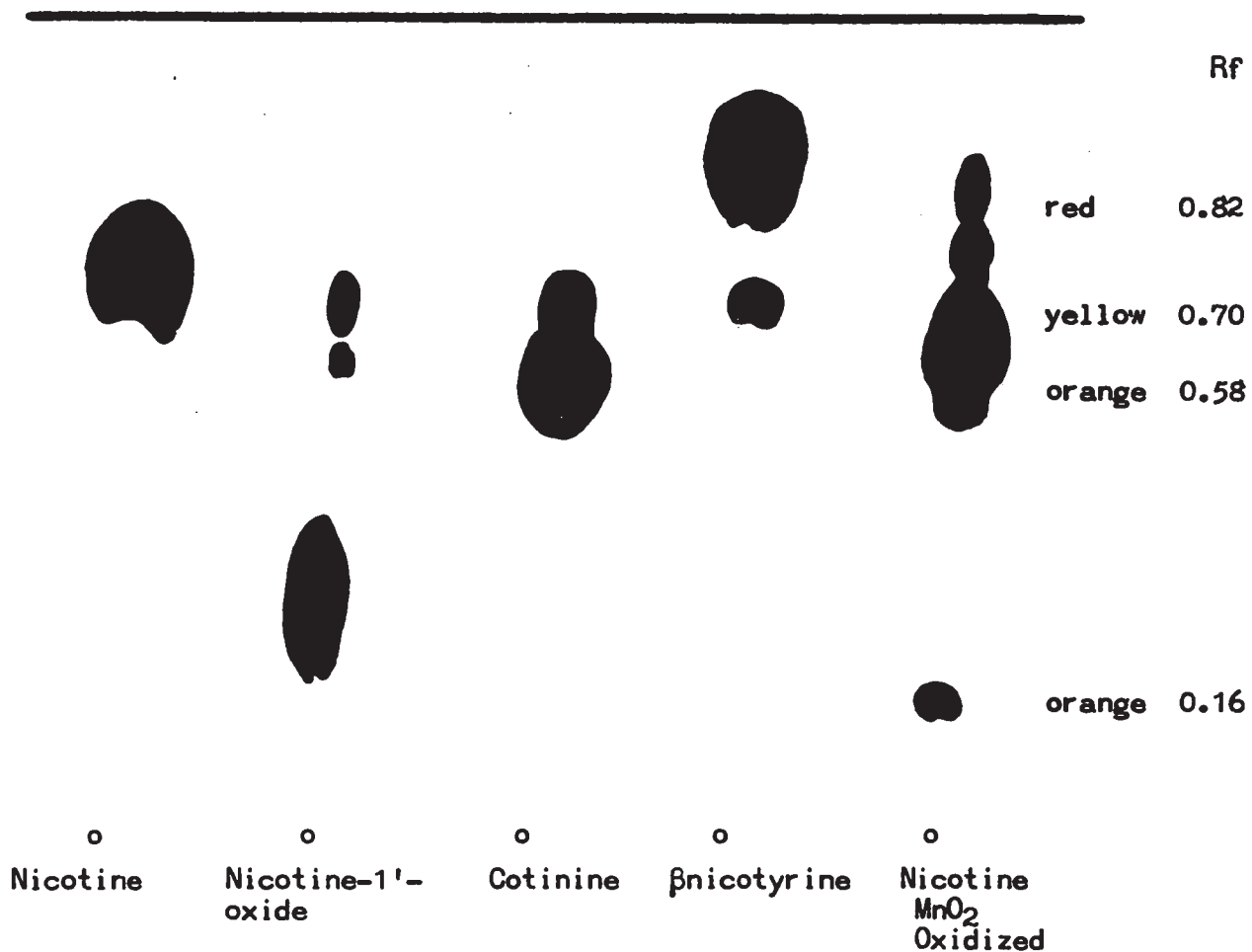


FIGURE 11

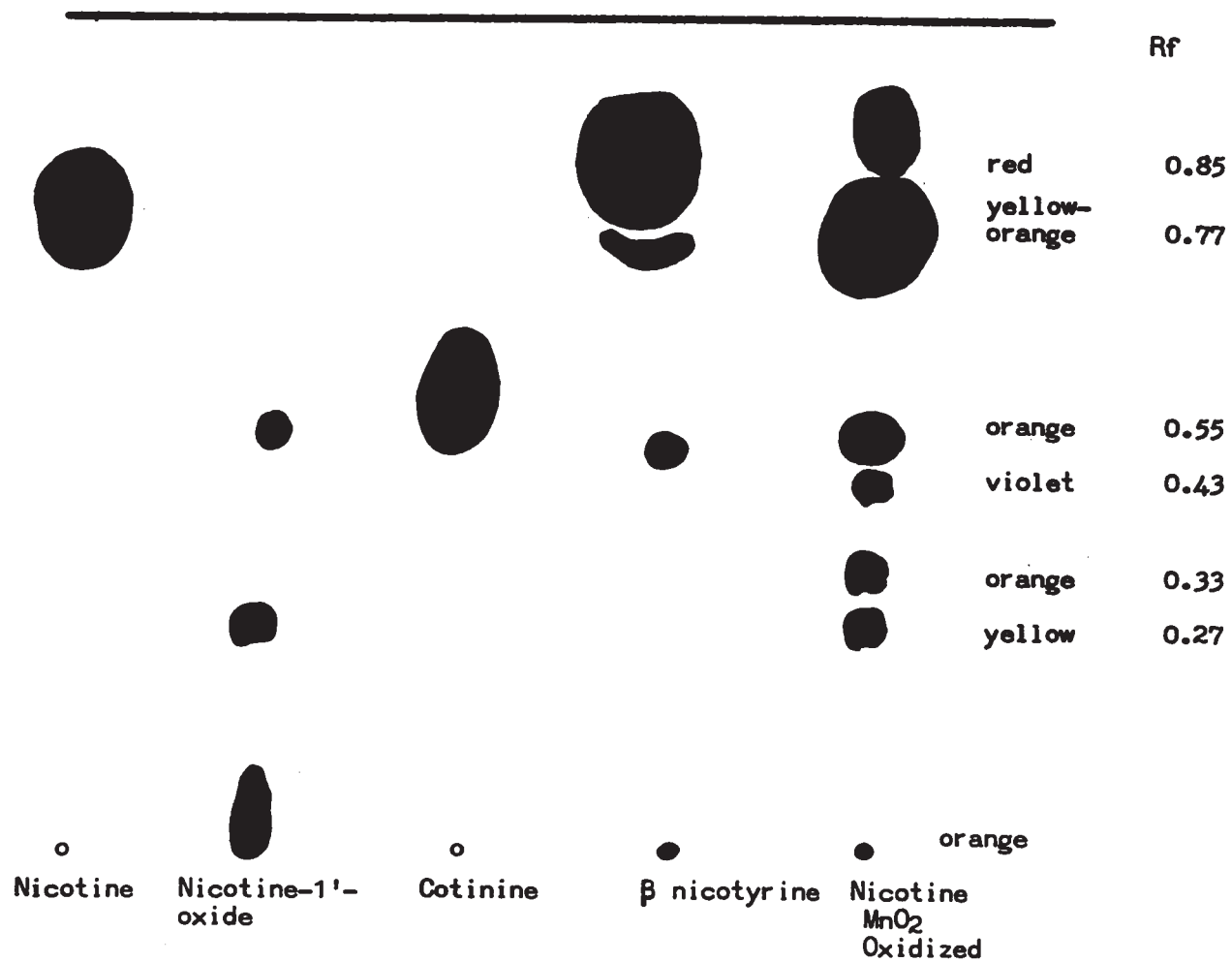
Thin Layer Chromatogram with Materials Spotted on
Silicar TLC-7G, Silica Gel Plates 250 Microns Thick,
Developed with 0.5 N NH_4OH , EtOH and n-Butanol (1:1:4);
the Spots Being Visualized by Spraying the Plate with 1%
para-amino Benzoic Acid, Then Exposing it to CNBr Vapour.



Solvent: 0.5N NH₄OH, EtOH and n-Butanol (1:1:4)

FIGURE 12

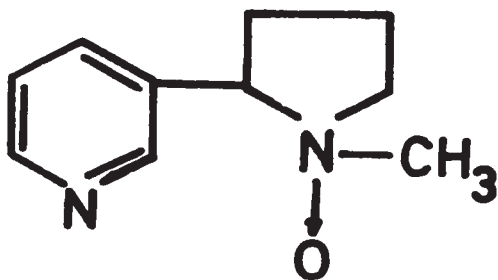
Thin Layer Chromatogram with Materials Spotted on
Silica TLC-7G Silica Gel Plates 250 Microns Thick,
Developed with Acetone, Benzene, EtOH and conc. NH_4OH
(40:50:5:5); the Spots Being Visualized by Spraying the
Plate with 1% para-amino Benzoic Acid, then Exposing it to CNBr Vapour.



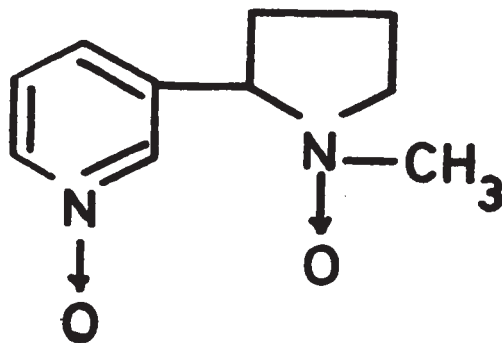
Solvent: Acetone, Benzene, EtOH and conc NH₄OH (40:50:5:5)

FIGURE 13.

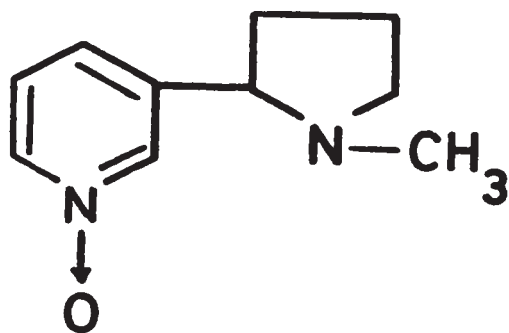
Three Possible nicotine-N-oxides
as Reported by Taylor and Boyer (1959).



Nicotine-1'-oxide



Nicotine-1,1'-dioxide



Nicotine-1-oxide

Magnetic Resonance.⁽¹⁾ Here it was confirmed by two analyses that the sample contained a mixture of nicotine-1'-oxide and nicotine-1-oxide in the approximate proportions of 2:1 as well as containing approximately 25 per cent water. The presence of cotinine in the sample was not detected in either of these N.M.R. analyses.

The results obtained from testing these nicotine oxides plus nicotinic acid, for their tremorogenic potency in mice both before and after DFP are presented in Table I.

At the end of this study, throughout which the mixture designated as nicotine-N-oxide was used, it was possible to obtain pure samples of nicotine-1'-oxide dihydrochloride⁽²⁾ and nicotine-1-oxide sulphate⁽³⁾. Testing was carried out on the tremorogenic effect of each of these compounds in mice, both alone and when given after DFP. The results of these tests are presented in Appendix VIII. Here it is indicated that the effect of DFP potentiated tremor was only evident with nicotine-1'-oxide.

(1) Courtesy Dr. M.A. Weinberger, Defence Research Establishment Ottawa and Dr. D.J. Currie, Defence Research Establishment Suffield.

(2) & (3) Synthesized by Dr. A.H. Gray, Defence Research Establishment Suffield.

TABLE I
Effect of D F P induced mouse brain cholinesterase depression
on the tremor produced by intracerebrally injected
nicotine and nicotine oxidation products

Drug	Dose response (no D F P)			Dose response (with D F P)			D.F P tremor potentiation
	Dose mg/kg	r	$\overline{ED_{50}}^{(1)}$	Dose mg/kg	r	$\overline{ED_{50}}^{(1)}$	
Nicotine	0.60	10/10		0.26	10/10		
	0.12	7/10	0.051	0.13	7/10	0.056	
	0.06	5/10	(0.026-0.096)	0.056	4/10	(0.029-0.11)	nil
	0.03	4/10		0.028	3/10		
Nicotine-N-oxide	100.0	10/10		10.0	9/10		
	28.2	8/10	14.0	5.0	8/10		
	20.0	6/10	(8.9-21.6)	1.0	8/10	0.29	est. 48 x
	10.0	5/10		0.3	4/10	(0.11-0.74)	
	5.0	1/10		0.1	3/10		
β nicotine				0.03	2/10		
				0.01	1/10		
	43.5	3/10 - 2/10 dead		43.5	4/10 - 6/10 dead		nil
	13.0	2/10		13.0	1/10		
Cotinine	4.4	3/10		4.4	1/10		
	2.0	1/10					
	100.0	0/10		100.0	0/10		nil
Nicotinic acid	50.0	8/10 - 7/10 dead		25.0	8/10 - 7/10 dead		nil
	20.0	8/10 - 3/10 dead		5.0	4/10		
	5.0	5/10		1.0	1.10		
	1.0	1/10					
	0.5	1/10					

⁽¹⁾With 95% confidence limits where calculated

G. The Tremorogenic Effect of Intracerebrally Injected
nicotine-N-oxide in Mice

The results of Table I indicated that of the nicotine oxidation products tested, that only with the sample designated as nicotine-N-oxide were the tremorogenic effects potentiated by previous DFP treatment. The dose response lines for tremor and/or convulsions following i.c. injection of nicotine and nicotine-N-oxide with and without DFP pretreatment are shown in Figure 14. Here the same phenomenon as observed with MnO_2 oxidized nicotine occurred in that DFP pretreatment caused a significant flattening of the dose response curve for nicotine-N-oxide as well as its shift to the left. Following this observation, nicotine-N-oxide was tested in mice pretreated with DFP, atropine, mecamylamine or hemicholinium-3. The effects of these various drug treatments upon the ED_{50} s for tremor and/or convulsions are illustrated in Figure 15, with the corresponding 95 per cent confidence limits for each ED_{50} value. The data yielding these results are tabulated in Appendix IX. Where parallelism between the dose response lines yielding these ED_{50} s was proven, relative potencies between each were calculated.

The results in Figure 17 indicated that pretreating the mice with atropine at 15.0 mg/kg was shown to have no effect upon the tremorogenic potency of nicotine-N-oxide. Mecamylamine at 1.7 mg/kg however, offered significant protection ($p < 0.05$) to the tremor. This was equal to 2.7X the ED_{50} of the nicotine-N-oxide alone. When this same dose of mecamylamine was given to mice which were also treated with a $1/2 \text{ LD}_{50}$ dose of DFP the protective effect of the mecamylamine

FIGURE 14

Dose Response Lines for Tremor and/or Convulsions
in Mice by i.c. Injection of Nicotine and nicotine-N-oxide
Given Alone, or 30 Minutes Following Intravenous D F P

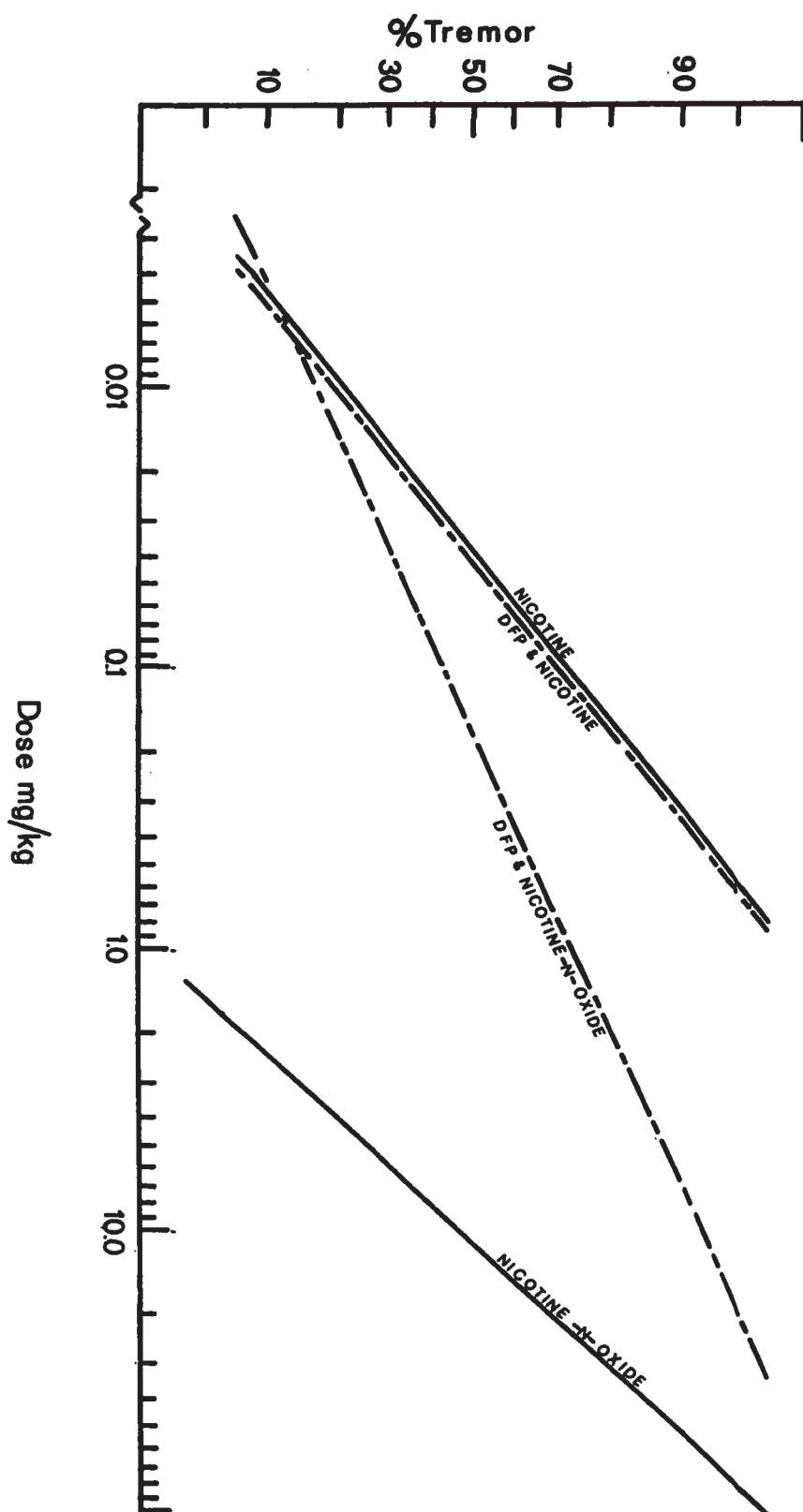
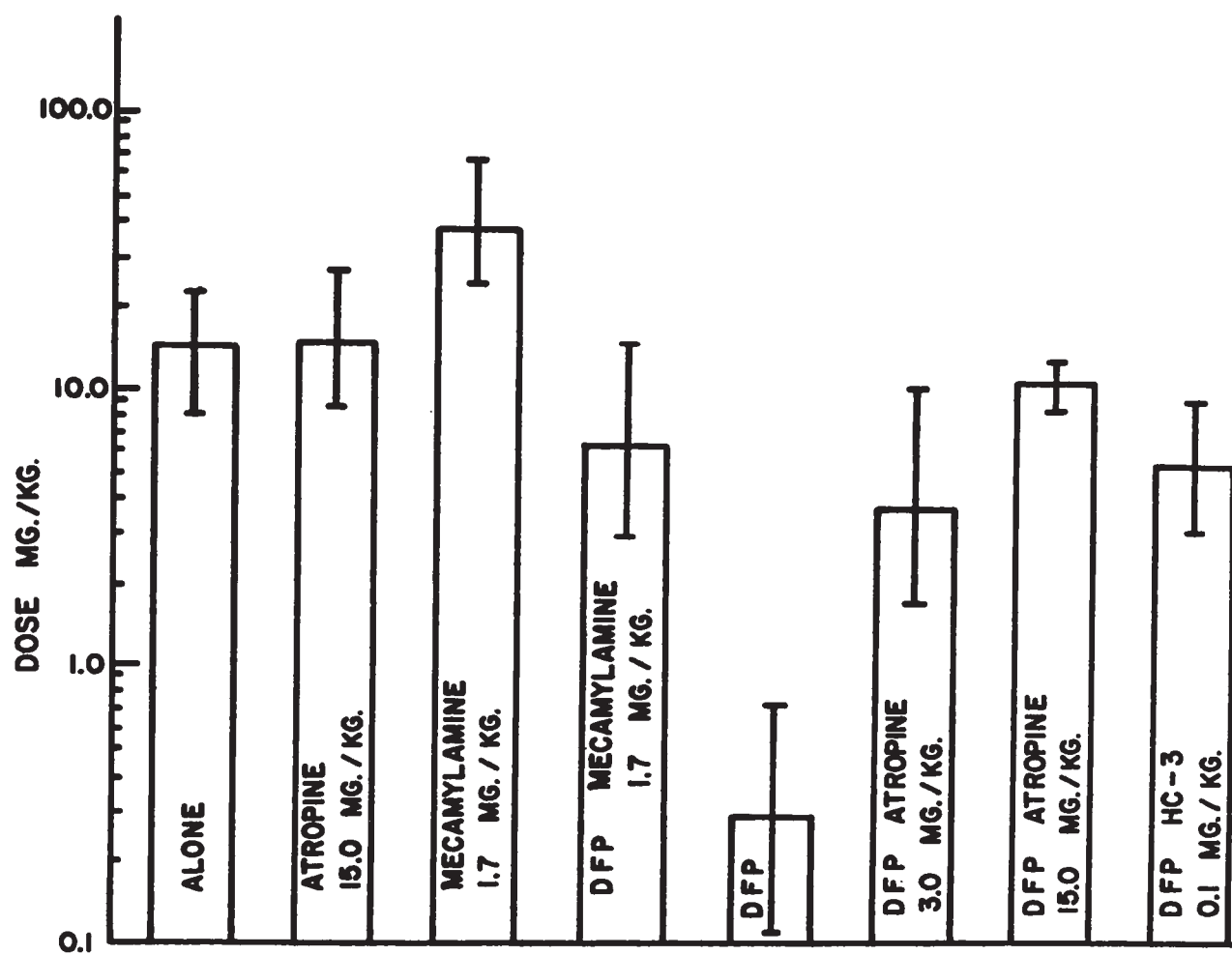


FIGURE 15

ED₅₀s and Their Corresponding 95 per cent Confidence Limits for Tremor and/or Convulsions Following i.c. Administration of Nicotine-N-oxide to Mice, Alone and 30 Minutes After Treatment with Atropine 15.0 mg/kg; Mecamylamine 1.7 mg/kg; DFP Plus Mecamylamine 1.7 mg/kg; DFP; DFP Plus Atropine 3.0 mg/kg; DFP Plus Atropine 15.0 mg/kg and One Hour after Hemicholinium-3 0.1 mg/kg Plus DFP Given 30 Minutes Before the Nicotine-N-oxide.



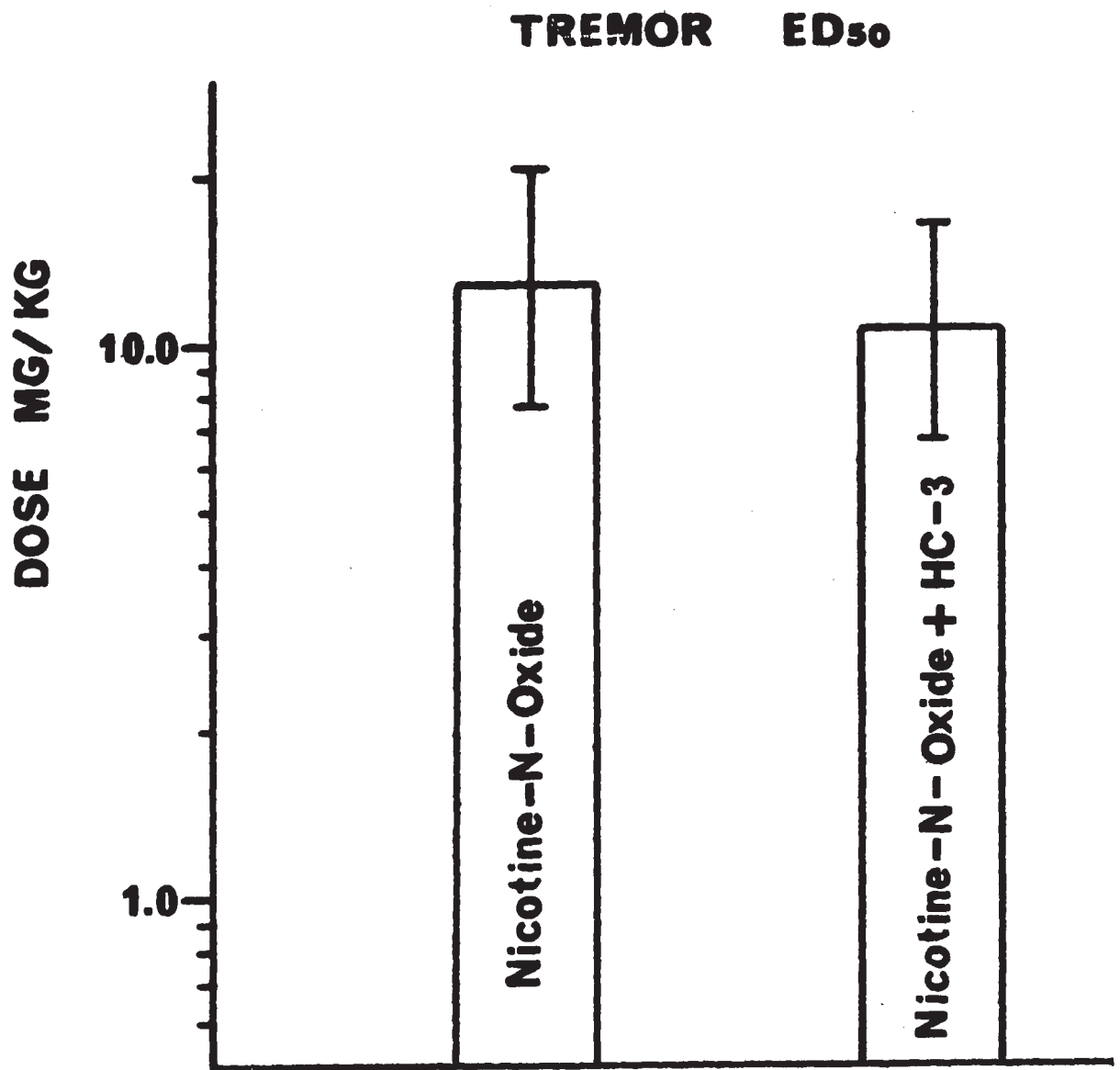
was reduced. Parallelism of the resulting dose response curves under these two different treatments was not proven, however the ED_{50} for tremor and/or convulsions of nicotine-N-oxide when only mecamylamine was given was 37.8 mg/kg and was reduced to 6.88 mg/kg when the mecamylamine was given to the DFP treated mice. This showed a 5.5X reduction in the mecamylamine protection which is similar to the results obtained when MnO_2 oxidized nicotine was given as the tremorogenic agonist.

As was previously shown in Figure 14, pretreating the mice with $1/2 LD_{50}$ of DFP 30 minutes prior to challenging them with an i.c. injection of nicotine-N-oxide greatly potentiated the nicotine-N-oxide effect. These particular dose response lines were proven to not be parallel, therefore the parametric methods of relative potency analysis were not carried out. In this case, however, a direct comparison revealed that the ED_{50} for tremor of nicotine-N-oxide after DFP pretreatment was reduced to 48X that which it was prior to the DFP pretreatment (Table 1). Atropine at a dose of 3.0 mg/kg partially removed this potentiating effect while a dose of 15.0 mg/kg completely removed the DFP potentiation. The effect produced by pretreating the mice with hemicholinium-3 was similar to that of atropine in that the potentiating effect of the DFP upon the nicotine-N-oxide tremor is almost completely removed by this drug.

The effect of HC-3 treatment alone upon the tremorogenic potency of nicotine-N-oxide was tested. Figure 16 shows the ED_{50} s and their 95 per cent confidence limits for nicotine-N-oxide and nicotine-N-oxide administered one hour after 0.1 mg/kg of HC-3.

FIGURE 16

ED₅₀s and Their Corresponding 95 per cent Confidence Limits for Tremor and/or Convulsions Following the i.c. Administration of nicotine-N-oxide to Mice, Alone and One Hour After i.c. Treatment with Hemicholinium-3 0.1 mg/kg.



The dose response lines for these two treatments were parallel and relative potency analysis revealed that there was no effect of this treatment upon the tremorogenic potency of the nicotine-N-oxide. The data from which these ED_{50} s were calculated appear in Appendix X.

The original suggestion advanced by Armitage et al (1966), Armitage and Hall (1967b) and Armitage et al (1969) was that nicotine is able to cause the release of Ach in the central nervous system. Further investigations were thus carried out in an attempt to determine if the apparent muscarinic response produced by nicotine-N-oxide in the presence of depressed brain acetylcholinesterase could be due to this action.

H. The Effect of DFP Pretreatment Upon the Level of Whole Brain Acetylcholine Recovered After Saline or Nicotine-N-Oxide with and without DFP Treatment, in Frozen and Unfrozen Brain Samples.

Initially it was desired to determine the maximum whole brain Ach levels one might expect to recover from CF-1 female mice used for the Ach brain level studies following the different treatments employed. Some brain samples were prepared by treating the mice by i.c. drug injection and after 3 minutes decapitating them, allowing the head to fall into liquid nitrogen. The head was allowed to remain in the liquid nitrogen for 10 seconds (sufficient to freeze the outside and cool the interior of the brain); then the brain was removed and transferred to the ice cold acidified ethanol stages of Ach extraction according to the method of Crossland and Slater (1968). The levels of whole brain Ach obtained by this method as assayed by the frog rectus abdominis muscle, compared to the levels obtained in the non-frozen brains, as a summation of their "free" and "bound" Ach levels, appear in Table 2.

TABLE 2

Total Brain Acetylcholine Levels in CF-1 Female Mice,

mM/g¹ Tissue Wet Weight \pm S.E.

Drug Treatment i.c. Injection	Frozen Brains		Non-Frozen Brains (Pooled "free" & "bound")	
Saline	15.6 \pm 2.1	n = 4	10.5 \pm 1.5	n = 8
Nicotine-N-oxide 20 mg/kg	12.9 \pm 2.0	n = 4	11.0 \pm 2.1	n = 8
Saline 30 min. after 1/2 LD ₅₀ DFP	22.4 \pm 1.0	n = 7	22.3 \pm 2.8	N = 8
Nicotine-N-oxide 20 mg/kg 30 min. after 1/2 LD ₅₀ DFP	19.2 \pm 1.2	n = 7	19.6 \pm 2.4	n = 8

The results of this investigation indicate that the intracerebral injection of a dose of nicotine-N-oxide sufficiently high to cause tremors in 70 per cent of the mice treated (20.0 mg/kg), did not significantly change ($p > 0.05$) the total brain Ach level from that found in mice which received only saline; both in cases where the brains were frozen prior to extraction and when they were not. This same non-significant effect of the nicotine-N-oxide was found in mice which had been pretreated with 1/2 LD₅₀ of DFP.

The effect of DFP in increasing total brain Ach levels was very evident, however, with the increase ranging from 1.4 to 1.5X in the frozen brains to 1.8 to 2.1X in the instance of the non-frozen brains. The effect of freezing in preventing the loss of Ach occurring due to extraction procedures is evident in the observation

¹One mM = One nM or 0.146 mg Ach.

of lower levels of recoverable Ach in non-frozen brains over frozen brains from mice which were not pretreated with DFP. This finding of the effect of freezing upon total Ach recovered is in agreement with what was reported by Crossland (1951). When the mice were pretreated with DFP, however, there was practically identical recovery of Ach in the frozen or unfrozen brains. This indicated the level of enzyme inhibition as a result of the DFP treatment, successfully prevented the loss of Ach occurring during the extraction procedure employed in unfrozen brain tissue.

1. The Effect of Nicotine-N-Oxide Upon the "Free" and "Bound" Level of Ach in Whole Mouse Brains

The mean levels of "free" and "bound" Ach in the brains of mice which were given no treatment, i.e. injection of saline or various doses of nicotine-N-oxide; alone or thirty minutes following an intravenous injection of $1/2$ LD₅₀ of DFP are shown in Figure 17. The data presented in this figure indicates that there was no significant change in the level of "free" or "bound" Ach between mice receiving no treatment at all and those given i.c. injections of saline or nicotine-N-oxide at 20.0 mg/kg. Mice which were pretreated with the $1/2$ LD₅₀ dose of DFP 30 minutes before receiving an i.c. injection of saline showed a doubling in the total brain Ach level. These results indicate significant increased in both the level of "free" ($p < 0.05$) and "bound" ($p < 0.01$) Ach occurring as a result of the DFP pretreatment, however the bulk of the increase is reflected in the level of the "bound" fraction. The results presented in Figure 17 also show that when nicotine-N-oxide was administered by i.c injection at the different doses of 20.0 mg/kg, 0.6 mg/kg and 0.02 mg/kg after DFP,

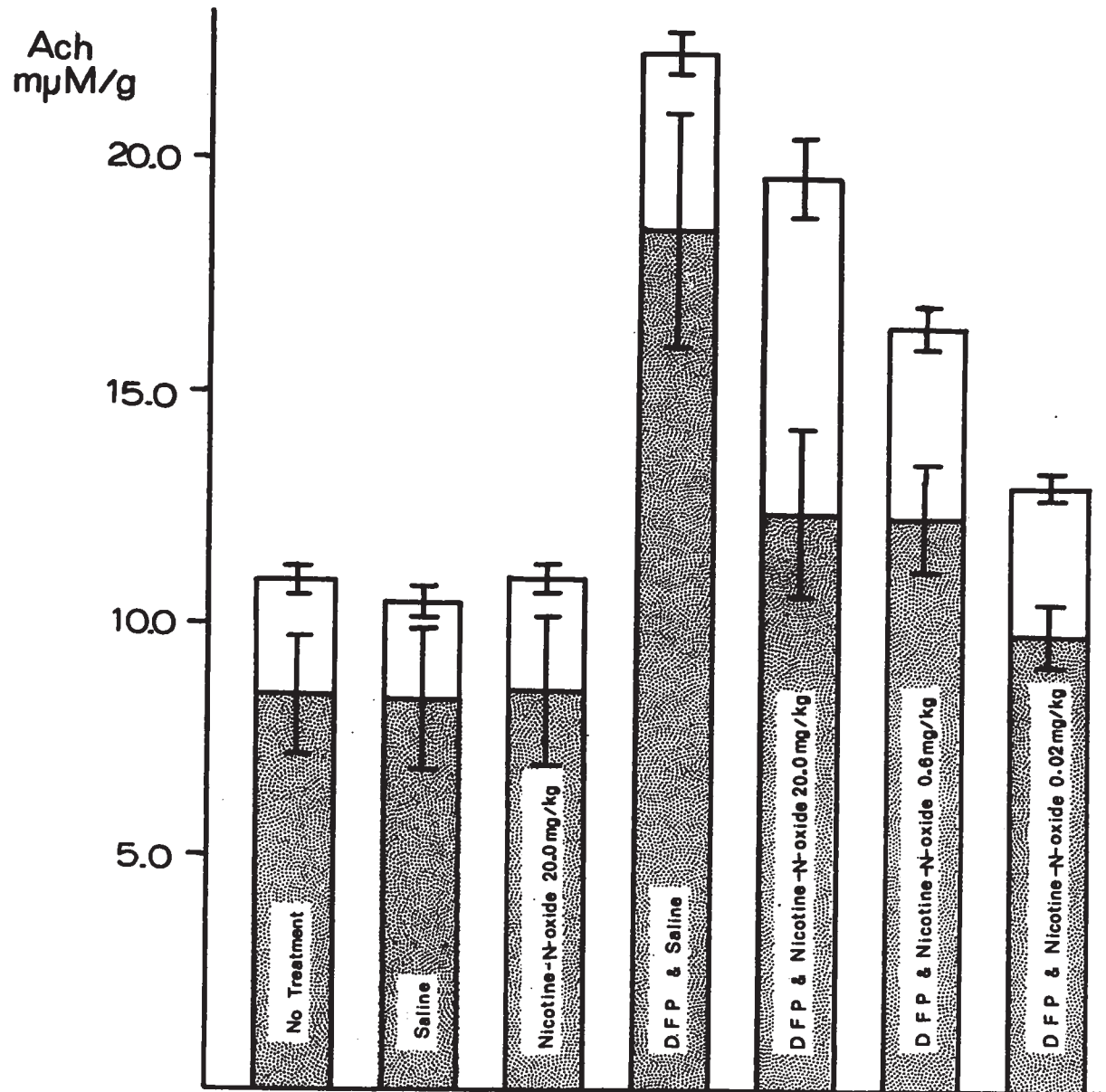
FIGURE 17

Mean Levels of "Free" and "Bound" Ach Plus Their Corresponding Standard Errors After no Treatment or i.c. Injection of Saline; nicotine-N-oxide 20.0 mg/kg; and 30 Minutes Following DFP of Saline; nicotine-N-oxide 20.0 mg/kg, 0.6 mg/kg, and 0.02 mg/kg.

"free" Ach 

"bound" Ach 

ACETYLCHOLINE LEVELS IN MOUSE BRAIN 3 MINUTES AFTER I.C. INJECTION



the "bound" fraction of Ach was reduced in all cases to a level that was not significantly different from the "bound" Ach found in the saline control. There was, however, a significantly higher level of "free" Ach noted after all three doses than found in the saline control ($p < 0.05$), with a dose response effect being noted.

J. The Effect of Nicotine Upon the "Free" and "Bound" Level of Ach in Whole Mouse Brains

A similar picture to what has been presented after nicotine-N-oxide injection was found in the case of nicotine. These results are shown in Figure 18. In the instance of intracerebrally injected nicotine, however, when given alone at a tremorogenic dose, 0.1 mg/kg, it was found that the level of "bound" Ach was significantly reduced over that found in mice which had received no treatment or an i.c. injection of 0.9 per cent saline, ($p < 0.01$). The level of "free" Ach was not found to be significantly different from normal in this instance. This effect of nicotine thus resulted in a significant lowering of the total brain Ach recovered, ($p < 0.05$).

In the case of nicotine, the two doses tested following DFP treatment resulted in a reduction in the "bound" Ach fraction with it returning to the control level at the lower dose administered. This was accompanied by significantly higher than normal levels of "free" Ach in both cases, ($p < 0.05$).

The relationships between the levels of "free" Ach measured, relative to the tremor dose response curves obtained with nicotine and nicotine-N-oxide, in mice given DFP and no DFP are shown in Figure 19. The mean levels of "free" Ach measured at the doses of nicotine or nicotine-N-oxide given are plotted on a log scale of $m\mu M/g$

FIGURE 18

Mean Levels of "Free" and "Bound" Ach Plus Their Corresponding
Standard Errors After no Treatment or i.c. Injection of Saline;
Nicotine 0.1 mg/kg and 30 Minutes Following DFP of Saline;
nicotine 0.1 mg/kg and 0.02 mg/kg.

"free" Ach 

"bound" Ach 

ACETYLCHOLINE LEVELS IN MOUSE BRAIN 3 MINUTES AFTER I.C. INJECTION

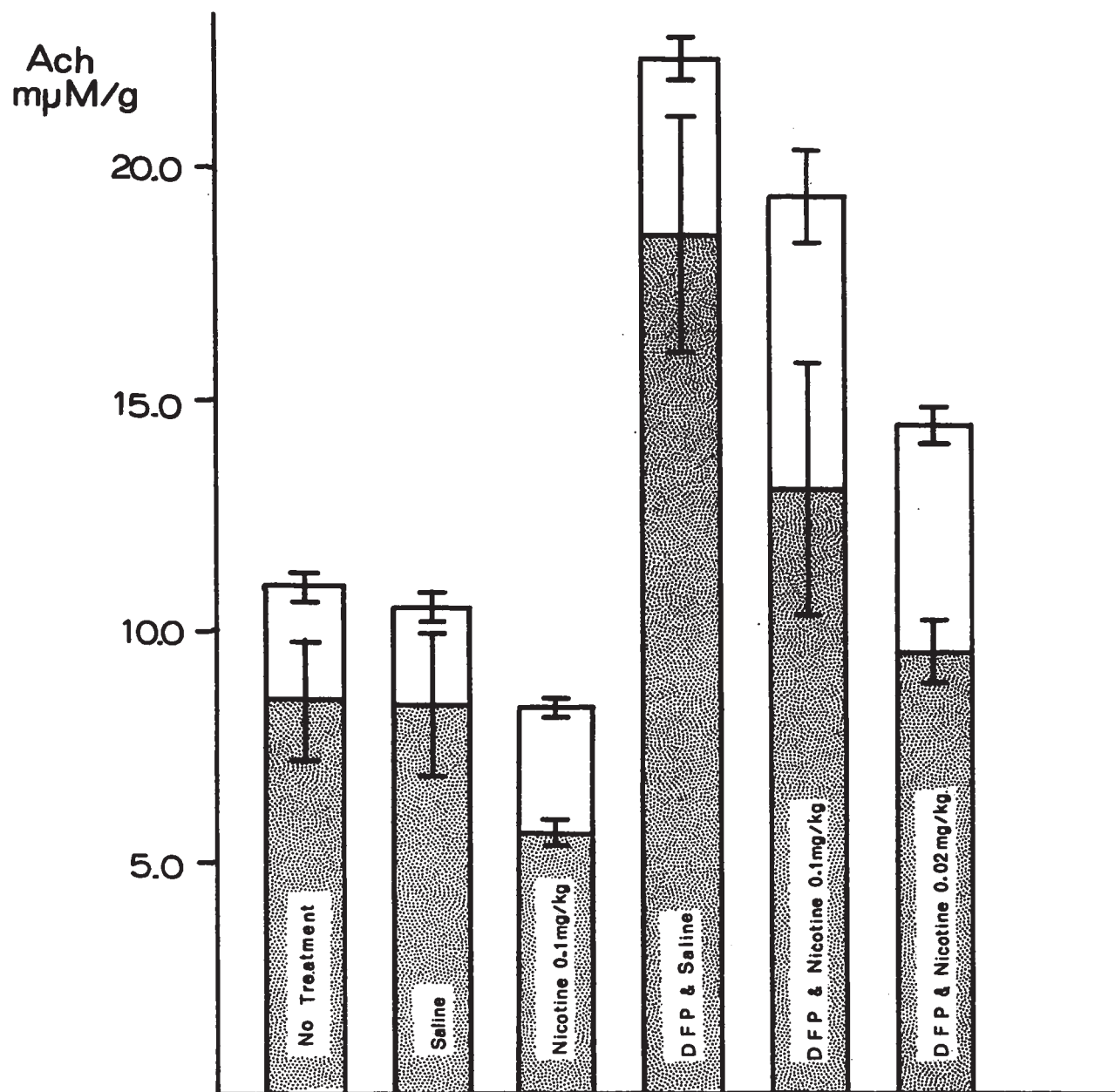
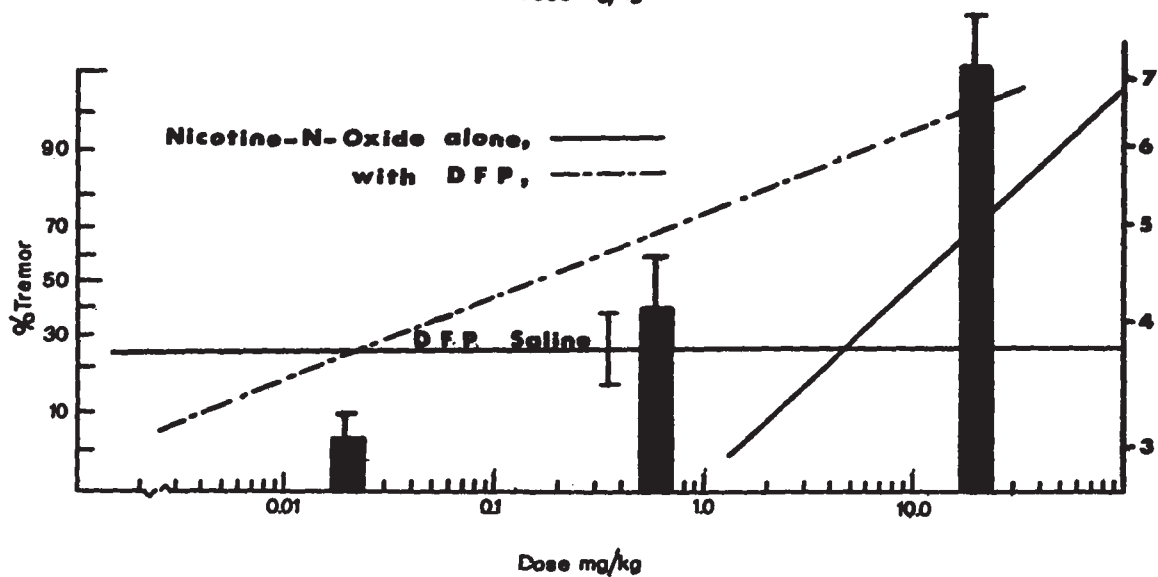
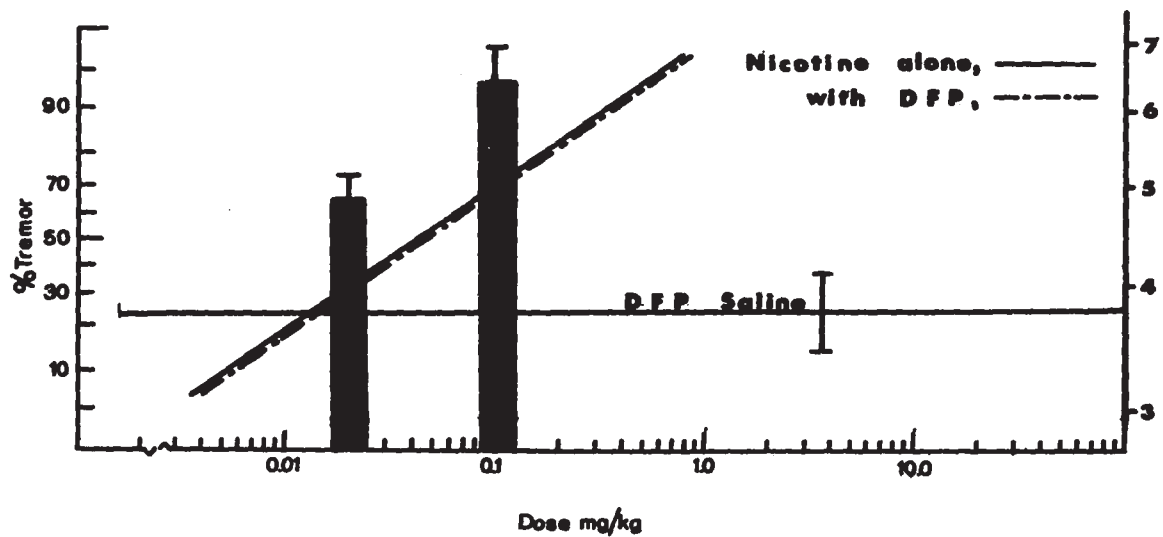


FIGURE 19

The Mean Levels of "Free" Ach Plus Their Corresponding Standard Errors in Mouse Whole Brains After i.c. Injections of Nicotine and Nicotine-N-Oxide Following DFP Pretreatment, Compared With the Dose Response Lines for Tremor and/or Convulsions of these Two Drugs When Given Alone and 30 Minutes After DFP.

TREMOR DOSE RESPONSE IN MICE AFTER I.C. INJECTION

Ach μ M/g



of tissue wet weight, with their corresponding S.E.s. These are compared to the level of "free" Ach measured in mice which were given saline after DFP.



This figure indicates that the level of "free" Ach recovered appears to be directly related to the dose of either nicotine or nicotine-N-oxide administered. The levels of "free" Ach do not bear a close relationship to the tremor dose response curve obtained from nicotine-N-oxide administered to DFP treated mice.

K. The Effect of Atropine and DFP Pretreatment on the Level of "Free" and "Bound" Acetylcholine in Whole Mouse Brains

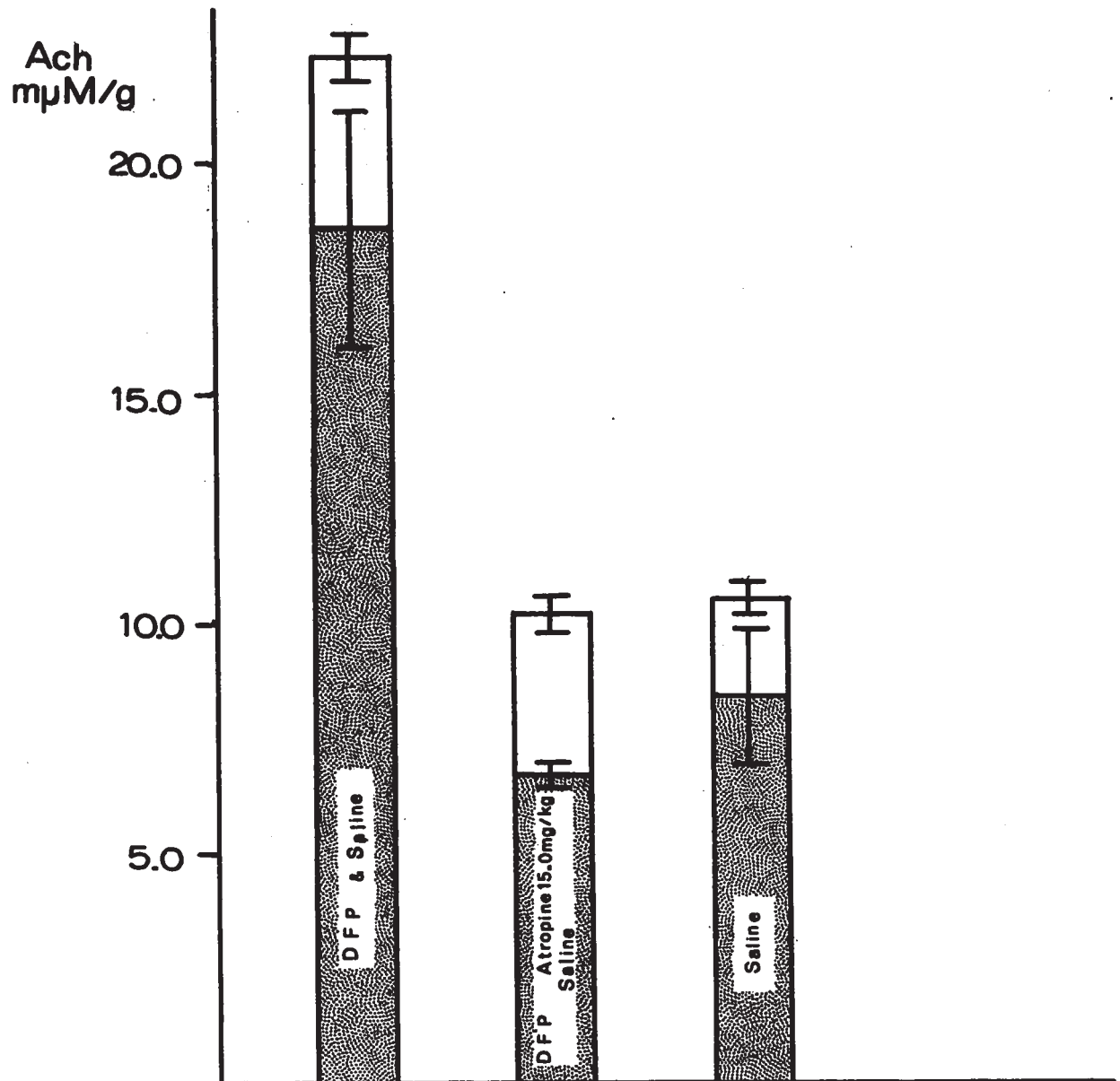
The effect of the administration of atropine, 15 mg/kg at the same time as DFP, upon the "free" and "bound" levels of Ach, is illustrated in Figure 20. These results indicate that atropine prevented the high "bound" level of Ach from occurring as a result of the DFP pretreatment. In fact the level of "bound" Ach measured appeared less than that recovered in mice which only received a saline injection. This difference is not significant however, ($p > 0.05$). The level of "free" Ach found after the atropine-DFP treatment was significantly higher than that measured in the saline treated mice ($p < 0.001$), however, it remained almost identical to the level of "free" Ach in mice given saline after DFP alone.

FIGURE 20

Mean Levels of "Free" and "Bound" Ach Plus Their Corresponding
Standard Errors After an i.c. Injection of Saline
Given Alone; 30 Minutes After DFP and DFP
Plus 15.0 mg/kg of Atropine.

"free" Ach 
"bound" Ach 

ACETYLCHOLINE LEVELS IN MOUSE BRAIN 3 MINUTES AFTER I.C. INJECTION



V. DISCUSSION

A. The Effect of Brain Cholinesterase Depression Upon the Central Tremorogenic Effects of Acetylcholine, Nicotine, Oxidized Nicotine and Nicotine Oxides

The dose of DFP used throughout this study ($1/2$ i.v. LD_{50}) produced a very pronounced effect in depressing brain acetylcholinesterase yet the mice thus treated did not show the tremorogenic or convulsive advanced symptoms of anticholinesterase poisoning. This finding is in agreement with the effects of this agent as reported by Frawley et al (1952) where it was demonstrated that the administration of a single sublethal dose, orally to rats produced only "minimal symptoms" when the brain acetylcholinesterase was depressed to an average level of 8 per cent. Frawley and his fellow workers clearly showed this was not the case with the other anticholinesterases which they studied and reported severe symptoms of poisoning occurred at much higher levels of functional brain acetylcholinesterase with the anticholinesterases; Parathion, tetraethylpyrophosphate (TEPP), octamethyl pyrophosphoramidate (OMPA), diethoxy thiophosphoric acid ester of 7-hydroxy-4-methyl coumarin (E-838) and ethyl p-nitrophenyl thionobenzene phosphonate (EPN).

DFP was also the agent of choice in this investigation for a number of other reasons. It could be purchased from a commercial source in a highly purified form and its purity could be readily monitored by frequent bioassessment for its lethal effects in mice. DFP is an irreversible inhibitor of cholinesterase (Mazur and Bodansky, 1946) thus would produce a uniform level of cholinesterase depression over a prolonged period of time. This agent is also reported not to have the action similar to eserine of inhibiting Ach uptake and binding, in brain tissue, as reported by Polak (1969) or release from the cortex (Szerb, 1964). It was thus believed DFP would therefore not interfere directly with brain Ach binding or release.

From the results obtained by the intracerebral injections of Ach, nicotine, oxidized nicotine and nicotine-N-oxide it was consistently shown that atropine was without effect in blocking the tremor produced by the nicotinic agonists used, yet was very effective in this regard against Ach. At the same time mecamylamine offered significant protection against the tremor produced by the nicotinic agonists but was without effect in blocking these similar effects produced by Ach. The finding that atropine was ineffective in blocking the tremor and/or convulsions induced by the nicotine compounds tested, is in agreement with the results reported by Holmstedt and Lundgren (1967) where it was reported that doses of atropine, even as high as 100 mg/kg, were unable to block the tremor produced by nicotine in rats. The central anti-muscarinic effects of atropine are well known however and are reflected in the ability of this drug to protect mice against the tremorogenic effects produced by intracerebrally injected Ach.

Mecamylamine, on the other hand, has been shown to be a potent centrally acting nicotinic antagonist (Stone et al, 1958; Knapp and Domino, 1962; Oliverio, 1966; and Morrison et al, 1969), while at the same time it is reported by Stone et al (1958), that Stone and Reyner in unpublished data found that this drug and other anti-nicotinics exhibited little atropine-like activity upon the effects of Ach on isolated rabbit intestine. Domino (1967) also reported that mecamylamine was effective in blocking doses of nicotine which produced an E.E.G. arousal response yet was ineffective in blocking a similar response evoked by the muscarinic drug arecoline. Mecamylamine may be viewed, within the context of this study, as exerting the effect of primarily blocking central nicotinic responses. This specificity is further supported in the work of McKinstry and Koelle (1967), where it was shown that mecamylamine does not inhibit the release of Ach induced by carbachol or preganglionic stimulation from the cat superior cervical ganglion.

The results obtained in this study show that the tremorogenic potency of auto-oxidized nicotine, MnO_2 oxidized nicotine and nicotine-N-oxide is potentiated by DFP induced brain acetylcholinesterase depression. It appears that this effect observed with oxidized nicotine is due to the presence of nicotine-N-oxide in the mixture. This is in agreement with what one would expect from the quantitative yield of oxidation products found to be present in aerated nicotine by Wada et al (1959), where it was reported that nicotine-N-oxide was present far in excess of any of the other oxidation products produced. Also nicotine-N-oxide was found to be present in the MnO_2 oxidized nicotine as indicated by chromatographic separation of this mixture. Furthermore, of the oxidation products tested, only nicotine-N-oxide produced potentiated

tremorogenic effects in brain acetylcholinesterase depleted animals. Additional biological testing done at the end of this study gives a strong indication that the specific nicotine-N-oxide producing this effect is nicotine-1'-oxide.

The DFP induced potentiation of the tremorogenic effects of nicotine-N-oxide are not attributed to this compound itself acting as a cholinesterase inhibitor. It was reported by Ghandi (1968) that the sample of nicotine-N-oxide used in this study did not show any inhibiting effect upon the acetylcholinesterase in mouse whole blood at a concentration of $1 \times 10^{-3}M$. This is in agreement with the relatively low anticholinesterase property attributed to nicotine. It was reported by Bain (1950), that nicotine would produce a 50 per cent inhibition of brain acetylcholinesterase at $5.0 \times 10^{-3}M$ concentration in a brain homogenate.

These results suggest that nicotine-N-oxide may act to produce tremor and/or convulsions by at least two different mechanisms. One of these may be attributed to its direct acting nicotinic agonistic action which is effectively blocked by mecamylamine but not atropine or HC-3; and the other appears to be a muscarinic component of action which becomes evident in animals with depressed brain cholinesterase. This latter effect of nicotine-N-oxide may be removed by pretreating the animals with atropine or HC-3 and causes a reduction in the effectiveness of the mecamylamine blockade. All the indirect pharmacological evidence obtained from this study points toward nicotine-N-oxide having a similar component of action as nicotine in that it causes Ach release.

The Ach releasing effect of nicotine is supported in the literature by Day and Vane (1963); Takagi et al (1967); Armitage and Hall (1967b); Morrison (1968); Henderson et al (1968); Chiou and Long (1969); Corley and Hoff (1969); Armitage et al (1969) and Morrison et al (1969). In this study, all the pharmacological evidence using oxidized nicotine and nicotine-N-oxide, administered to mice treated with DFP; atropine; mecamlamine or HC-3 supports this action for nicotine-N-oxide as well. The one exception is found in the instance of nicotine given to mice which had been pretreated with DFP; or DFP and mecamlamine. Based upon the finding that mecamlamine was ineffective in protecting DFP treated mice against Ach and the observations that the protective effect of mecamlamine in DFP treated mice was reduced for oxidized nicotine and nicotine-N-oxide, it is reasonable to assume one should record a similar decrease in the case of DFP treated mice given nicotine. This was not observed. A possible explanation for this effect may lie in the fact that very high doses of nicotine were administered to produce tremor and/or convulsions following the treatment combination of mecamlamine and DFP. Phillis and York (1968), in studying the effect of nicotine and Ach on the spontaneous and induced firing of cortical neurones, report an inhibiting effect attributed to both compounds with nicotine being much more potent than Ach in producing this effect. It is thus possible that the doses of nicotine, sufficiently high to produce tremors and/or convulsions in mecamlamine treated mice, would cause the effects of any Ach released to be unobserved. A similar type of explanation may be advanced for the observation that brain acetylcholinesterase depression did not further potentiate the tremorogenic potency of pure nicotine. The highly potent nicotinic agonistic properties

of pure nicotine, in doses sufficient to produce tremor, masked the effects of any Ach which they release.

A possible explanation for the potentiation of the tremor produced by oxidized nicotine and nicotine-N-oxide after DFP yet not nicotine may lie in the potency difference between the treatments. In this study it was found that nicotine is 270X more potent a tremorogenic than nicotine-N-oxide when injected i.c. in mice and 25X more potent in causing contractions of the frog rectus abdominis muscle. Bizard et al (1956) reported that the subcutaneous toxicity difference in mice between nicotine and nicotine-1'-oxide was 20X, while the potency difference in the guinea pig ileum is 70X and that nicotine is 50 to 100X more active as a ganglionic stimulant than nicotine-1'-oxide. Arutyunyan and Mashkovski (1961), found nicotine to be 20X more potent in causing contractions of the rabbit intestine than nicotine-1'-oxide. They also reported the acute toxicity of nicotine to be 50X i.v.; 31X s.c. and 6.3X orally more toxic than nicotine-1'-oxide. Yamamoto and Domino (1965) demonstrated that nicotine was 30X more potent in producing a pressor response and E.E.G. behavioral arousal in cats than nicotine-N-oxide.

From the results of this study it is concluded that both nicotine and nicotine-N-oxide have a qualitatively similar type of action to produce tremor. This is reflected in the parallelism of the two dose response lines for tremor for nicotine and nicotine-N-oxide and the effectiveness of mecamylamine and ineffectiveness of atropine in protecting animals against both. Even though the potency difference of nicotine-N-oxide is much less as a direct acting "nicotinic" agonist this compound appears to retain the property of nicotine in being able to cause Ach release. There exists the possibility that

nicotine-N-oxide may be only a partial agonist relative to nicotine. There is no evidence in this study or the literature to support this. If this were so it could well attribute to the enhanced muscarinic response observed in DFP treated mice due to Ach mobilization. The parallelism of dose response curves for nicotine and nicotine-N-oxide may give some support to this not being the case, however, a more critical comparison of the dose response action of this compound on several isolated organ preparations should be carried out. The tremor occurring in brain acetylcholinesterase depressed mice by nicotine-N-oxide, at doses which are below those which would cause tremor in normal mice is believed to be due to the mobilization of Ach. The effect of pretreating the mice with HC-3 by i.c. injection offers some evidence that Ach is the contributing factor to the potentiated tremor observed with nicotine-N-oxide in DFP treated mice. This treatment significantly removed this potentiation yet it was demonstrated to have no effect upon the tremorogenic potency of nicotine-N-oxide given alone.

B. The Effect of Nicotine and Nicotine-N-Oxide Upon "Free" and "Bound" Levels of Acetylcholine in Whole Mouse Brains

The measurement of "free" and "bound" Ach in whole mouse brains treated with i.c. saline, DFP plus i.c. saline or DFP followed by various i.c. doses of nicotine or nicotine-N-oxide, did not produce conclusive evidence to support the hypothesis that the potentiated tremorogenic effect of nicotine-N-oxide in DFP treated mice is due to Ach release.

The measurements of the total Ach levels found in mice 30 minutes after being given $1/2 \text{ LD}_{50}$ of DFP i.v. revealed a doubling in the total level of Ach measured. This finding is in agreement with other work conducted on cholinesterase inhibitors; (DuBois et al, 1949; Stewart, 1952; Robinson et al, 1954; Michaelis et al, 1954 and Holmstedt et al, 1967). The findings in this investigation demonstrate that the greatest proportional increase occurred in the "bound" fraction of brain Ach. Slater and Rogers (1968) also noted increases in "free" and "bound" Ach after treating rats with eserine; however, the total increase which they found was not as pronounced as in this study and their proportional increase in the "free" Ach fraction was higher than the increase in the "bound" fraction. This same picture was also presented by Crossland and Slater (1968). The higher proportional levels of "free" Ach found by these authors could well be attributed to the fact that they used eserine as the cholinesterase inhibitor. Polak and Meeuws (1966), Schubert and Sundewald (1967) and Polak (1969) have all reported that eserine is able to cause the release of Ach from bound stores. The fact that eserine was the inhibitor of acetylcholinesterase in the brain extraction procedure used in this study may similarly have resulted in an elevation of "free" Ach being measured. In future studies of this same nature it may be advisable to use a cholinesterase inhibitor such as DFP or Soman in the brain extraction of Ach rather than eserine. Many previous authors accept the view that there is no pre-formed "free" Ach in the normal animal thus the levels recorded in this study may be confounded by the effect of eserine causing some "bound" Ach to be displaced,

even during the extraction procedure. If this were the case, it would be advisable to use an inhibitor of acetylcholinesterase which does not contain a cationic moiety in the molecule. DFP or Soman are examples of such inhibitors as they are believed to bind to the cholinesterase molecule at the esteratic site only. These particular anticholinesterases are relatively unstable in solution at pH 7.4, however, it is possible to still use them. An extraction medium such as artificial C.S.F. would be recommended as well, as an imbalance of Na^+ , K^+ , and Ca^{++} will affect Ach release.

To detect subtle differences in "free" Ach measured, one would presumably have to use a more sensitive means of bio-assessment such as the dorsal muscle of the leech or perhaps abandon the bioassay and use one of the available gas chromatographic methods presently developed which may measure Ach in very minute amounts.

When nicotine was given to normal mice at a tremorogenic dose of 0.1 mg/kg, it was found that the level of "bound" Ach was significantly reduced over that found in normal mice or those which only received an i.c. injection of saline. This finding is in agreement with that reported by Pepeu (1965) who demonstrated that intravenously administered tremorogenic and convulsive doses of nicotine resulted in a decrease in total brain Ach in the rat when the determinations of brain Ach were done very soon after the nicotine injection, i.e. 20-60 seconds. Holmstedt and Lundgren (1967) reported no change in whole brain Ach after an i.p. administration of a tremorogenic dose of nicotine in the rat when determinations were done 3 and 10 minutes after the injection. The effects observed in this study and that of Pepeu (1965) may be attributed the more direct

route of administration used, which in both cases produced an almost immediate tremorogenic effect.

When i.c. doses of nicotine were given to DFP treated mice at doses of 0.1 mg/kg and 0.02 mg/kg (equivalent to the ED_{70} and ED_{30} tremorogenic doses respectively) it was found that there was a significant reduction in the "bound" Ach recovered with significant increases in "free" Ach being measured. The levels of "free" Ach recovered appear to be directly related to the tremorogenic potency of the dose of nicotine administered. A very similar pattern in the shift from "bound" to "free" Ach was measured following the administration of i.c. nicotine-N-oxide to DFP treated mice at doses of 20.0 mg/kg (ED_{70} tremorogenic dose), 0.6 mg/kg and 0.02 mg/kg (both sub-tremorogenic doses). Here, as was found with nicotine, there was a significant reduction in the "bound" Ach fraction after all three doses, however the levels of "free" Ach were more directly proportional to the tremorogenic potency of the dose of nicotine-N-oxide administered. Under the conditions of this experiment the levels of "free" Ach recovered following the nicotine-N-oxide injection to DFP treated mice do not give an indication of the potentiated tremor observed. The consistent reduction in the "bound" level of Ach recovered following all doses of both nicotine and nicotine-N-oxide, however do lend some support to an effect of these drugs being that of mobilizing Ach from its "bound" form.

If one is to assume that the potentiated tremor produced by nicotine-N-oxide is the result of mobilization of Ach from the high level of "bound" Ach found after DFP treatment, then the failure to recover more "free" Ach following the lowest doses of nicotine-N-oxide administered can only be a function of methodology.

Perhaps the failure to recover more of the original Ach released at the lower doses of nicotine-N-oxide is related to the time of decapitation (i.e. in this case 3 minutes after i.c. injection), since the tremor produced under these conditions appears within 30 seconds after treatment. Schuberth, Sparf and Sundewald (1969) have shown that there is a very rapid turnover of Ach in the brain of the mouse. If one is to assume that nicotine-N-oxide does not directly affect the mechanism of Ach synthesis, however may operate by displacing "bound" Ach, then the level of displaced Ach and the "bound" stores occupied by this nicotine cation could grossly alter the rate of Ach synthesis, (Bellamy, 1959; Giarman and Pepeu, 1962; Sharkawi and Schulman, 1969; and Kaita and Goldberg, 1969). Another possible explanation for this dose response failure to recover more "free" brain Ach may lie in a central component of nicotine-N-oxide action being like nicotine, in that it may have a primary initial effect of causing hypotension and bradycardia (Pradham, Bhattachaya and Atkinson, 1967; and Armitage and Hall, 1967a) and that the effect of a cholinesterase inhibitor potentiates the fall in blood pressure just as it does in the case of intra-ventricularly injected nicotine (Armitage and Hall, 1967a). This hypotensive effect may affect cerebral perfusion significantly thus contributing to one being able to recover higher levels of "free" Ach when the high doses of 20 mg/kg of nicotine-N-oxide or 0.1 mg/kg of nicotine were administered.

Either of these possibilities may contribute to the results obtained from the whole brain Ach measurements. It is to be emphasized however, that the cholinergic mechanism of synthesis, storage and release is a dynamic process in vivo and perhaps the only positive manner in which it should be studied is to take cognizance of this and measure Ach release from the brain of the living animal; similar to the methods used by Mitchell (1963); Szerb (1964); Polak and Meeuws (1966) and Armitage et al (1969).

The levels of "free" and "bound" Ach measured following the pretreatment of the mice with DFP and atropine present some evidence for an additional action of atropine to remove the potentiation of the nicotine-N-oxide tremor produced by DFP. Here it is shown that when atropine, 15.0 mg/kg i.p., is administered at the same time as the i.v. $1/2$ LD₅₀ of DFP, it prevents the rise in the "bound" fraction of Ach. This finding is reflected in the work of Holmstedt and Lundgren (1967) where they report that atropine given before, or at the same time as, was able to prevent the rise in total brain Ach in rats upon administration of the cholinesterase inhibitor F37A. Giarman and Pepeu (1964), Polak and Meeuws (1966) and Bertels-Meeuws and Polak (1968) have indicated that the release of Ach is stimulated by atropine and centrally active cholinolytics. These authors, as well as Szerb (1964) and Polak (1969) have postulated that atropine releases Ach from the brain in the living animal by blocking its re-absorption or by displacing it from its stores. The present finding on the profound reduction caused by atropine on the "bound" acetylcholine fraction found after DFP administration supports this theory. It is also to be assumed that atropine would in addition, exert a postsynaptic blocking action to the effects of the released Ach.

The action of nicotine and nicotine-N-oxide in causing a shift from the high level of "bound" stored Ach to Ach in the "free" form could very well occur by a similar mechanism as proposed for atropine and other centrally active cholinolytics in that these cations may displace Ach from its areas of storage. The physiological observance of the results of this action are made present in animals with depressed brain acetylcholinesterase using a compound like nicotine-N-oxide which is neither a strong enough cholinergic agonist or antagonist to mask the effects of the released Ach. The low level of brain acetylcholinesterase activity allows for a buildup of "bound" Ach for ready displacement as well as the reduction in the hydrolysis of the released Ach, therefore allowing its physiological effects to be manifested in the form of a potentiated tremor.

VI. CONCLUSIONS

1. Depression of brain acetylcholinesterase to a level of 9.4 ± 0.17 per cent of normal by $1/2 LD_{50}$ of i.v. injected DFP had no effect upon the tremorogenic potency of pure nicotine injected intracerebrally in mice.
2. Brain acetylcholinesterase depression by DFP resulted in the potentiation of the tremorogenic potency of auto-oxidized nicotine or nicotine which had been oxidized by MnO_2 equal to 6X and 10X respectively, the ED_{50} s for tremors and/or convulsions of these compounds given alone.
3. A similar potentiation of the tremorogenic response to an i.c. injection of MnO_2 oxidized nicotine was shown by pretreating the mice 30 minutes before with a $1/2 LD_{50}$ i.v. dose of the anti-cholinesterase inhibitor soman.
4. Of the auto-oxidation products tested only the tremorogenic effect of nicotine-N-oxide was potentiated by DFP brain acetylcholinesterase depression. This was equal to 48X the ED_{50} for tremor and/or convulsions of nicotine-N-oxide alone.
5. The potentiation by DFP upon the nicotine-N-oxide tremorogenic effect is attributed to nicotine-1'-oxide as the active constituent of the nicotine-N-oxide tested.

6. Atropine at 3.0 mg/kg was ineffective in blocking the tremorogenic effect of pure nicotine, however, an equi-molar dose of mecamlamine (1.7 mg/kg) conferred protection equal to 72X that of the ED_{50} for tremor of nicotine administered alone.
7. Atropine at a dose of 3.0 mg/kg was effective in partially removing the potentiating effect of DFP upon MnO_2 oxidized nicotine and nicotine-N-oxide tremor. DFP pretreatment significantly reduced the protection afforded by mecamlamine at 1.7 mg/kg to MnO_2 oxidized nicotine and nicotine-N-oxide.
8. Pretreating mice with 0.1 mg/kg i.c. hemicholinium-3 was effective in reducing the potentiating effect of DFP pretreatment upon the tremorogenic potency of nicotine-N-oxide, yet had no effect upon the tremorogenic potency of this compound given to non DFP treated mice.
9. Intracerebral injections of saline or saline containing 20.0 mg/kg of nicotine-N-oxide to non DFP treated mice produce no significant change in the "free" or "bound" Ach levels measured.
10. Pretreating the mice with $1/2 LD_{50}$ of DFP by i.v. injection resulted in a doubling of total brain Ach with the greatest proportional increase occurring in the "bound" fraction.
11. Treating mice with i.c. injections of nicotine-N-oxide 20.0 mg/kg, 0.6 mg/kg and 0.02 mg/kg 30 minutes after $1/2 LD_{50}$ DFP resulted in a reduction of the "bound" fraction of Ach to a level not significantly different from that found in the control mice. Significantly higher levels of "free" Ach were recovered with this effect being related to the dose of nicotine-N-oxide administered.

12. A tremorogenic dose of i.c. nicotine given alone significantly reduced the level of "bound" Ach over that found in normal mice while the level of "free" Ach remained the same.
13. Similar effects to those produced by nicotine-N-oxide were found by nicotine in DFP treated mice, in that doses of 0.1 mg/kg and 0.02 mg/kg resulted in a decrease in the "bound" fraction of Ach recovered with an accompanying rise in the "free" fraction found.
14. Atropine administered at 15.0 mg/kg i.p. the same time as the 1/2 LD₅₀ i.v. dose of DFP prevented the rise in the "bound" fraction of Ach which was associated with the DFP treatment alone.
15. Nicotine-N-oxide and nicotine appear to have similar qualitative activity in mobilizing brain Ach from the "bound" fraction in the brain. This property is believed to be responsible for the tremor potentiation by DFP pretreatment upon the effect produced by nicotine-N-oxide. The fact that this potentiation is not observed with nicotine yet is with nicotine-N-oxide is attributed to the much greater potency of nicotine as a direct acting nicotinic agonist.

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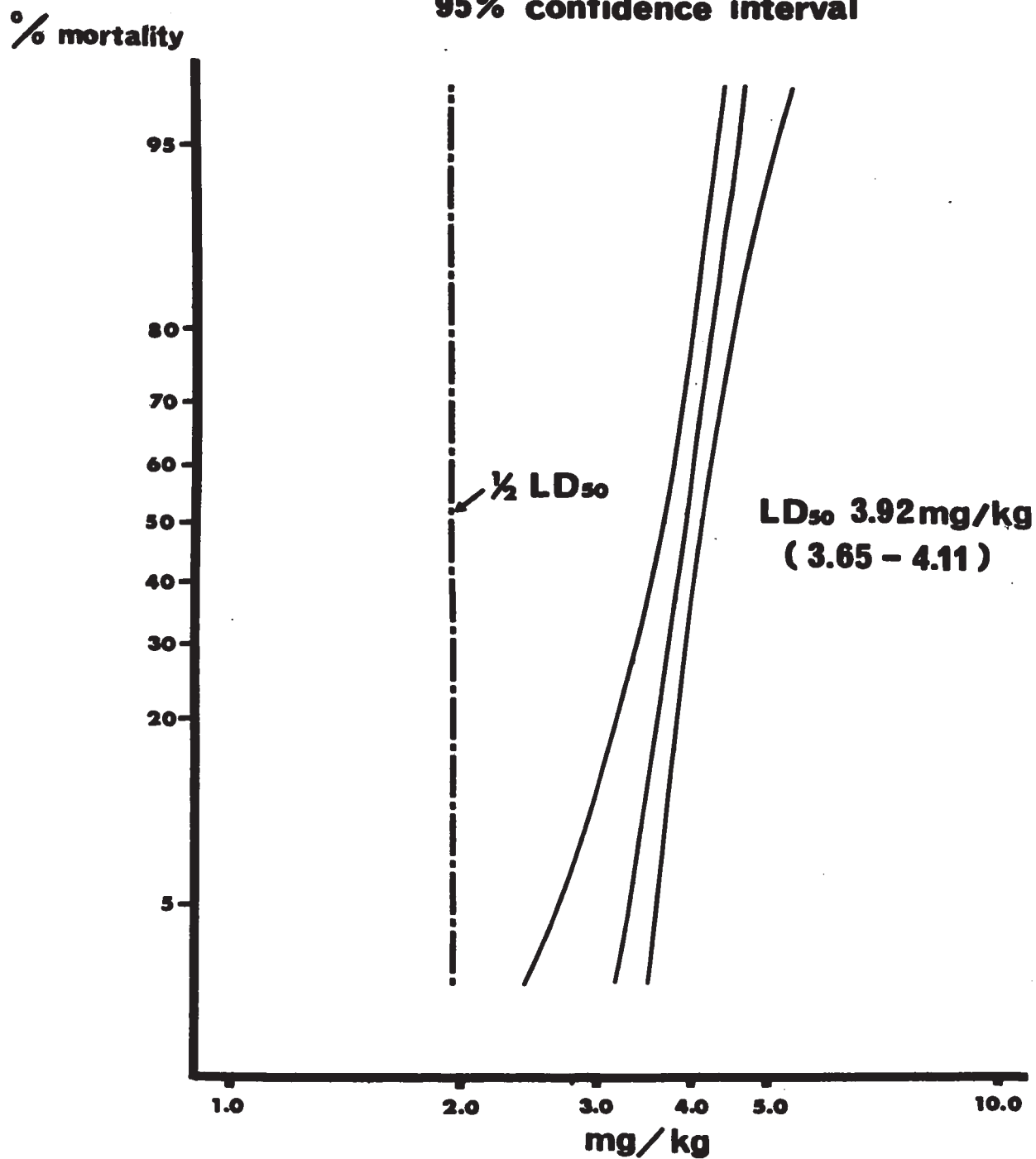
APPENDIX I

Dose response line for DFP toxicity in mice by i.v. injection with the
LD₅₀ and 95 per cent confidence interval

The purity of the sample of D.F.P. utilized was frequently sampled by i.v. toxicity in C.F.1 female mice weighing between 20-26g. The sample to be assayed was diluted in a 0.9% NaCl solution on the day of the assay and the assay was performed soon after the solution was prepared.

Mice, in groups of ten, were injected i.v. with selected doses of D.F.P. in the saline solution at an injection volume of 0.005 ml/g and an injection rate of 0.01 ml/sec. The groups of treated mice were housed in separate containers with food and water ad libidum. Observation for mortality was carried out over a 24 hr. period. A quantal response of dose mortality was recorded and used to calculate the dose response line with a minimum of four doses used in each assay. The dose response line and its corresponding ED_5 , ED_{20} , ED_{35} , ED_{50} , ED_{65} , ED_{80} and ED_{95} were calculated by the method of probit analysis (Finney 1947) and the line plus its corresponding 95% confidence interval was calculated.

i.v. DFP TOXICITY IN MICE
95% confidence interval



APPENDIX II

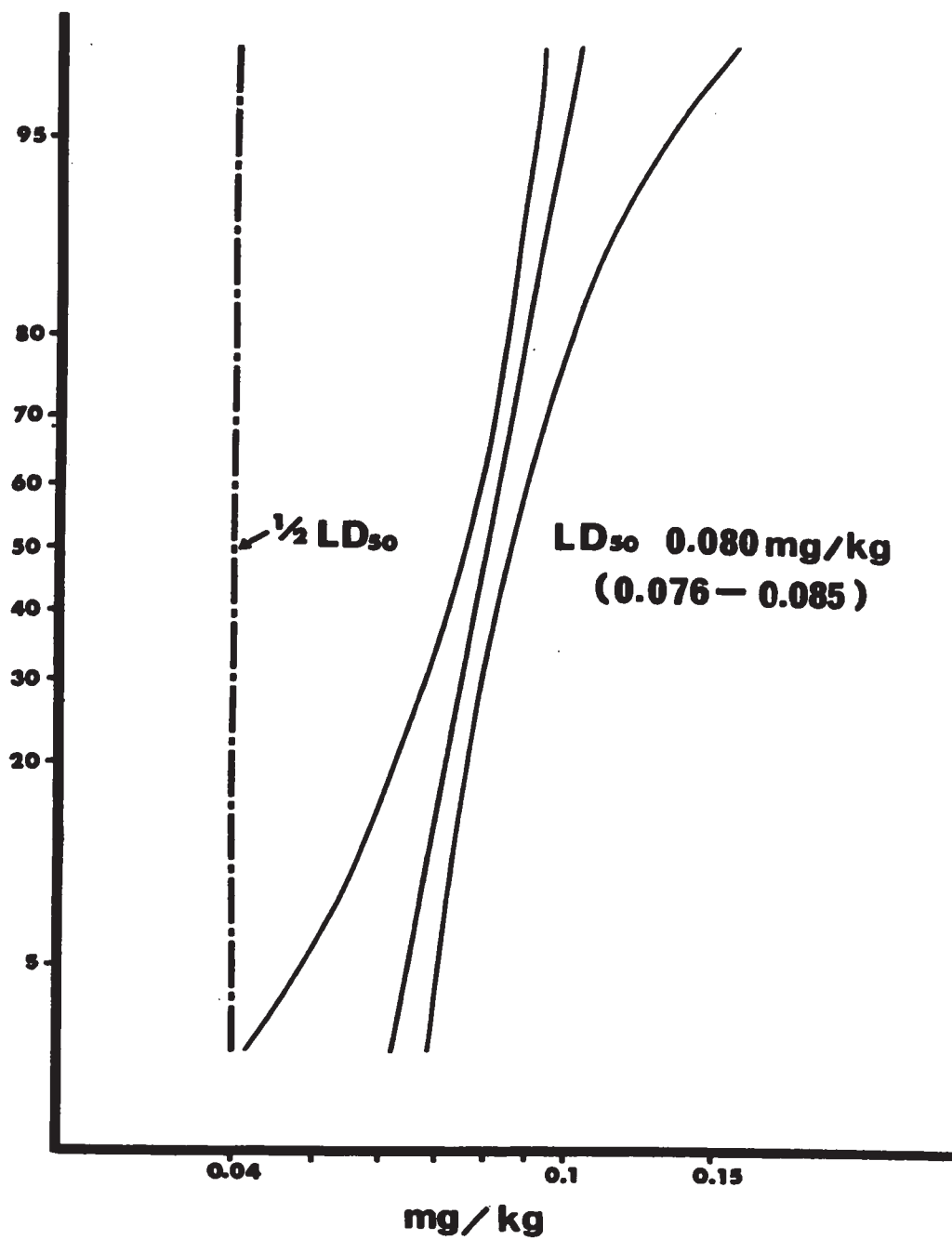
Dose response line for soman toxicity in mice by i.v. injection with the
LD₅₀ and 95% confidence interval about the line.

A single sample of soman was obtained courtesy the Defence Research Establishment Ottawa for this study. This sample was stored in a sealed glass container, packed in charcoal in a sealed metal container, which in turn was stored in a locked deep freeze. The purity of the sample was assayed by i.v. toxicity in female C₅₇F₁ mice weighing between 20-26g. The sample when assayed, was allowed to warm to room temperature and a v/v dilution made into a 0.9% NaCl solution. This was done on the same day as the assay, and the solution was assayed soon after the dilution was made.

Mice in groups of ten were injected i.v. with selected doses of the soman in the saline solution at an injection volume of 0.005 ml/g and an injection rate of 0.01 ml/sec. The groups of treated mice were housed in separate containers with food and water ad libidum. Observation for mortality was carried out over a 24 hr. period. A quantal response of dose mortality was recorded and used to calculate the dose response line with five doses being used. The dose response line and the corresponding ED₅, ED₂₀, ED₃₅, ED₅₀, ED₆₅, ED₈₀ and ED₉₅ were calculated by the method of probit analysis (Finney 1947) and the line plus its corresponding 95% confidence interval was calculated.

i.v. SOMAN TOXICITY IN MICE
95% confidence interval

% mortality



APPENDIX III

Dose response for tremor and or convulsions of i.c. ACh in mice⁽¹⁾

Drug treatment	Dose mg/kg	r	ED ₅₀ (95% confidence limits)
ACh	18.7	8/10	8.99 (4.25 - 15.5)
	10.7	6/10	
	8.0	4/10	
	5.4	3/10	
ACh 30 min after atropine 3.0 mg/kg. i.p.	107.0	9/10	13.2 (2.42 - 25.4)
	80.0	10/10	
	26.7	6/10	
	8.0	4/10	
ACh 30 min after 1/2 LD ₅₀ D F P i.v.	5.35	10/10	0.0948 (0.0381 - 0.245)
	0.535	8/10	
	0.177	5/10	
	0.0535	5/10	
	0.0177	2/10	
	0.00535	1/10	
ACh 30 min after 1/2 LD ₅₀ D F P i.v. plus atropine 3.0 mg/kg. i.p.	53.5	10/10	4.50 (1.75 - 8.58)
	16.1	8/10	
	5.35	6/10	
	1.61	2/10	
ACh 30 min after 1/2 LD ₅₀ D F P i.v. plus mecaml- amine 1.7 mg/kg.	2.70	10/10	0.057 (0.028 - 0.125)
	0.270	8/10	
	0.107	7/10	
	0.027	4/10	
	0.0107	1/10	
	0.0027	0/10	
ACh 30 min after atropine 3.0 mg/kg. i.p. (LD ₅₀)	160.0	8/10	LD ₅₀ 73.3 (32.0 - 131.0)
	107.0	6/10	
	80.0	5/10	
	26.7	2/10	

(1) Male mice 22-28g from the inbred colony maintained at the Defence Research Establishment Suffield.

APPENDIX IV

Dose response for tremor and or convulsions after i.c. Nicotine in mice

Drug treatment	Dose mg/kg	r	ED ₅₀ (95% confidence limits)
Nicotine alone	0.60	10/10	0.051 (0.012 - 0.099)
	0.12	7/10	
	0.06	5/10	
	0.03	4/10	
Nicotine 30 min after i.v. 1/2 LD ₅₀ of D F P	0.26	10/10	0.060 (0.029 - 0.099)
	0.13	7/10	
	0.056	4/10	
	0.028	3/10	
Nicotine 30 min after i.v. 1/2 LD ₅₀ of D F P and 30 mg/kg of atropine i.p.	0.50	9/10	0.081 (0.047 - 0.14)
	0.30	8/10	
	0.10	6/10	
	0.07	4/10	
	0.04	4/10	
	0.03	2/10	
	0.005	0/10	
Nicotine 30 min after 1.7 mg/kg. of mecamlamine i.p.	10.0	7/10	3.86 (2.11 - 9.93)
	6.0	6/10	
	4.0	5/10	
	2.0	4/10	
	1.0	2/10	
	0.5	1/10	
Nicotine 30 min after i.v. 1/2 LD ₅₀ of D F P and 1.7 mg/kg. of mecamlamine i.p.	10.0	7/10	5.57 (3.06 - 16.7)
	6.0	5/10	
	3.0	3/10	
	1.0	1/10	

APPENDIX V

Dose response for tremor and or convulsions after i.c. Air Oxidized Nicotine

Drug treatment	Dose mg/kg	r	ED ₅₀ (95% confidence limits)
Air oxidized	1.74	10/10	
nicotine alone.	0.87	8/10	
	0.43	5/10	0.29 (0.13 - 0.48)
	0.22	4/10	
	0.11	3/10	
Air oxidized	0.13	8/10	
nicotine 30 min	0.087	7/10	0.048 (0.016 - 0.090)
after 1/2 LD ₅₀	0.044	4/10	
of D F P	0.022	3/10	
Air oxidized	0.20	9/10	
nicotine 30 min	0.15	8/10	
after 1/2 LD ₅₀	0.10	7/10	0.086 (0.047 - 0.17)
of D F P and	0.07	5/10	
3.0 mg/kg of	0.05	2/10	
atropine	0.03	1/10	
Air oxidized	13.0	7/10	
nicotine 30 min	4.4	5/10	6.0 (3.0 - 18.6)
after 1.7 mg/kg.	1.8	1/10	
of mecamlamine.			
Air oxidized	10.0	10/10	
nicotine 30 min	8.0	7/9	
after 1/2 LD ₅₀	6.0	7/10	4.0 (2.9 - 5.2)
of D F P and 1.7	4.0	5/10	
mg/kg. of mecaml-	3.0	3/10	
amine.	2.0	2/10	

APPENDIX VI

Dose response for tremor and or convulsions after i.c. MnO₂ Oxidized
nicotine in mice

Drug treatment	Dose mg/kg	r	ED ₅₀ (95% confidence limits)
MnO ₂ oxidized nicotine	1.0	10/10	
	0.57	9/10	
	0.32	6/10	
	0.18	4/10	0.199 (0.135 - 0.296)
	0.10	2/10	
	0.057	2/10	
	0.032	0/10	
MnO ₂ oxidized nicotine 30 min after i.v. 1/2 LD ₅₀ of D F P	1.0	10/10	
	0.32	8/10	
	0.10	7/10	
	0.032	6/10	0.019 (0.006 - 0.050)
	0.010	3/10	
	0.0032	3/10	
	0.0010	2/10	
MnO ₂ oxidized nicotine 30 min after i.v. 1/2 LD ₅₀ of D F P and 3.0 mg/kg. atropine i.p.	1.0	9/9	
	0.32	8/10	
	0.10	6/10	0.067 (0.032 - 0.132)
	0.056	5/10	
	0.032	2/10	
	0.010	2/10	
MnO ₂ oxidized nicotine 30 min after 3.0 mg/kg. mecamylamine i.p.	13.8	10/10	
	11.8	8/10	
	10.9	2/10	11.3 (10.8 - 12.0) LD ₅₀
	10.0	1/10	
MnO ₂ oxidized nicotine 30 min after i.v. 1/2 LD ₅₀ of D F P and 3.0 mg/kg. mecamylamine i.p.	10.0	8/9	
	3.0	5/10	
	1.0	3/10	2.37 (1.14 - 5.85)
	0.33	1/10	

APPENDIX VII

Dose response for tremor and of convulsions after i.c. Nicotine and
oxidized nicotine in mice alone and following 1/2 LD₅₀ of
intravenous Soman

Drug treatment	Dose mg/kg	r	ED ₅₀ (95% confidence limits)
Nicotine	0.60	10/10	0.051 (0.012 - 0.099)
	0.12	7/10	
	0.06	5/10	
	0.03	4/10	
Nicotine 30 min after i.v. 1/2 LD ₅₀ of Soman	0.20	9/10	0.055 (0.032 - 0.102)
	0.10	6/10	
	0.05	4/10	
	0.025	4/10	
	0.015	1/10	
MnO ₂ oxidized nicotine	1.0	10/10	0.199 (0.135 - 0.296)
	0.57	9/10	
	0.32	6/10	
	0.18	4/10	
	0.10	2/10	
	0.057	2/10	
	0.032	0/10	
MnO ₂ oxidized nicotine 30 min after i.v. 1/2 LD ₅₀ of Soman	0.80	8/8	0.049 (0.013 - 0.109)
	0.30	7/10	
	0.15	5/8	
	0.075	6/10	
	0.030	5/10	
	0.010	2/10	

APPENDIX VIII

Tremorogenic effect of nicotine-1-oxide sulphate and nicotine-1'-oxide
dihydrochloride

Tremorogenic effect of nicotine-1-oxide sulphate and nicotine-1'-oxide dihydrochloride

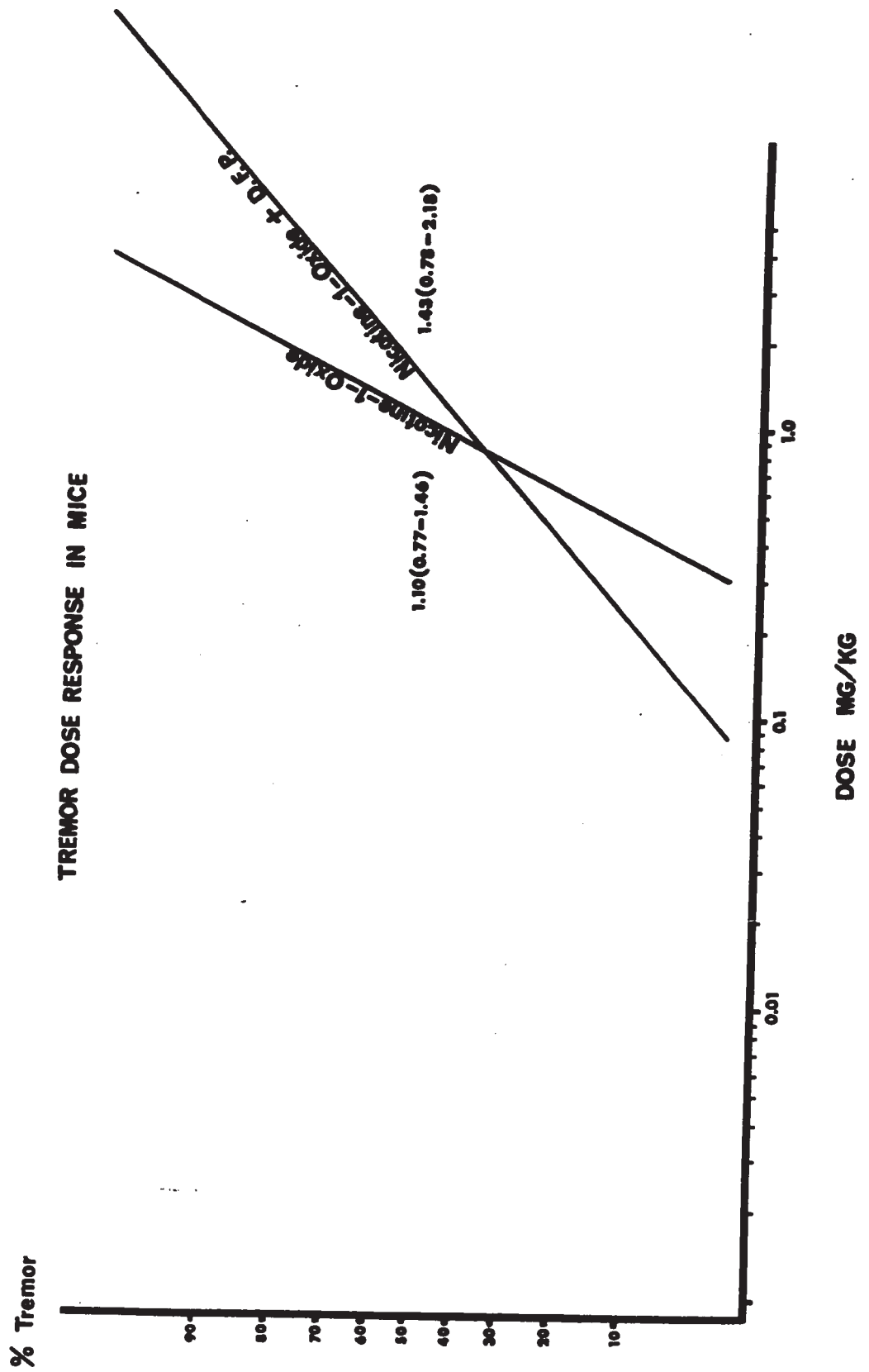
Upon completion of the major portion of the work reported in this thesis it became possible to secure pure samples of nicotine-1-oxide sulphate and nicotine-1'-oxide dihydrochloride. These were both tested for their tremorogenic potency by i.c. injection in normal mice and mice having been pretreated with $\frac{1}{2}$ LD₅₀ dose i.v. of D F P. The tremorogenic dose response results obtained for nicotine-1-oxide sulphate and nicotine-1'-oxide dihydrochloride are illustrated in Fig. I and II of this appendix. The resulting ED_{50's} for tremor of these two compounds given before and after D F P with their corresponding 95% confidence limits are shown in Fig. III of this appendix. The data obtained to produce these figures is presented in Table I.

These results indicate by relative potency analysis that nicotine-1-oxide sulphate is significantly 2.6x more potent a tremorogenic than nicotine-1'-oxide dihydrochloride (p0.05). This corresponds with a factor of 2.5x more potent in causing death in mice i.v. injection reported by Arutyunyan and Mashkovski (1961).

Pretreating the mice with D F P resulted in a significant flattening of the dose response line for tremor in the case of both of the nicotine oxides tested however only nicotine-1'-oxide dihydrochloride showed a potentiation in its tremorogenic potency due to cholinesterase depression. This amounted to a factor of 11.2x on the basis of inspection.

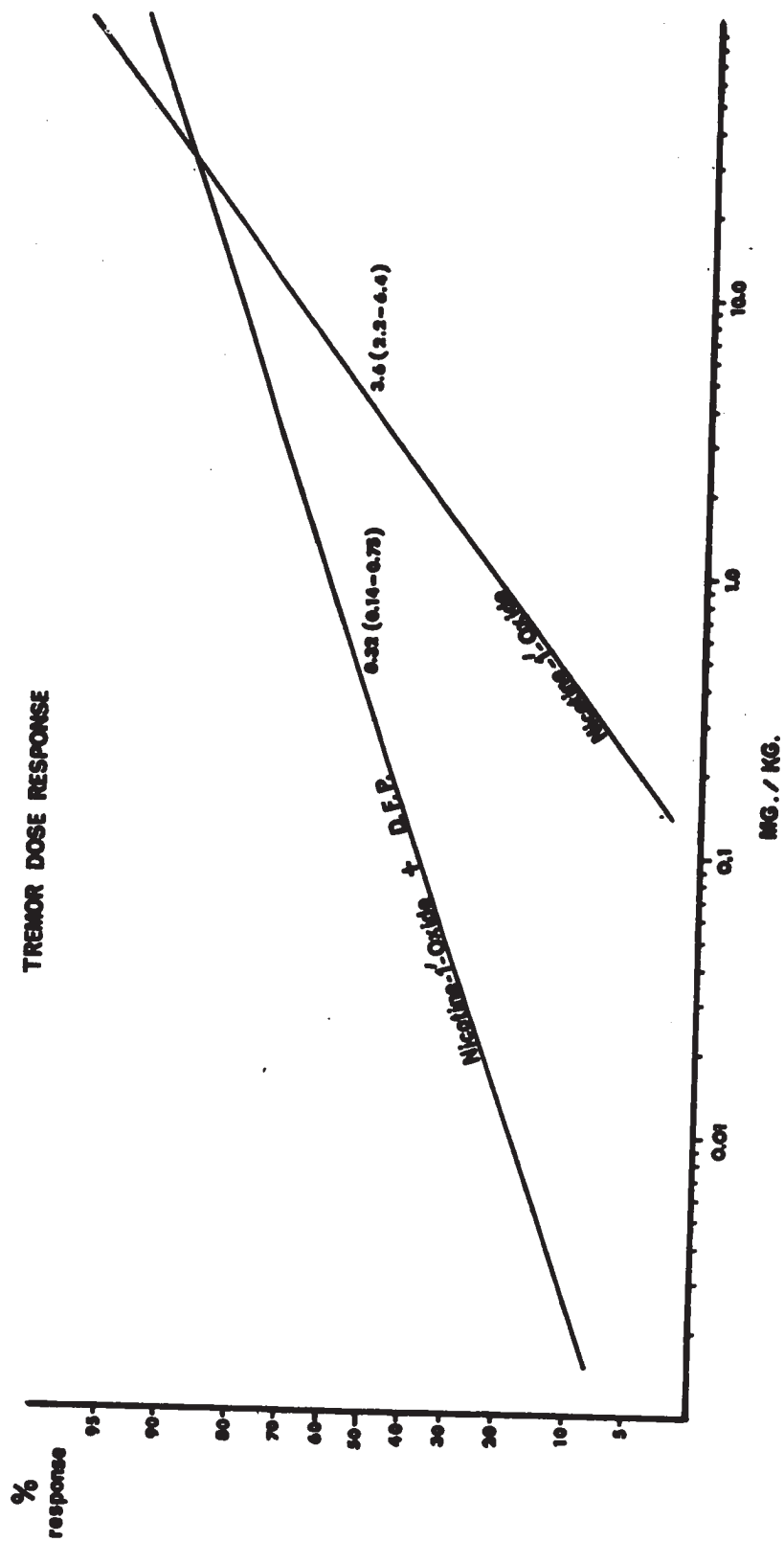
Appendix VIII

Fig.1 The dose response lines for tremor
and or convulsions with their ED_{50} 's following
i.c. injection of nicotine-1-oxide sulphate in
normal mice and mice pretreated with $\frac{1}{2}$ LD_{50}
i.v. of D F P



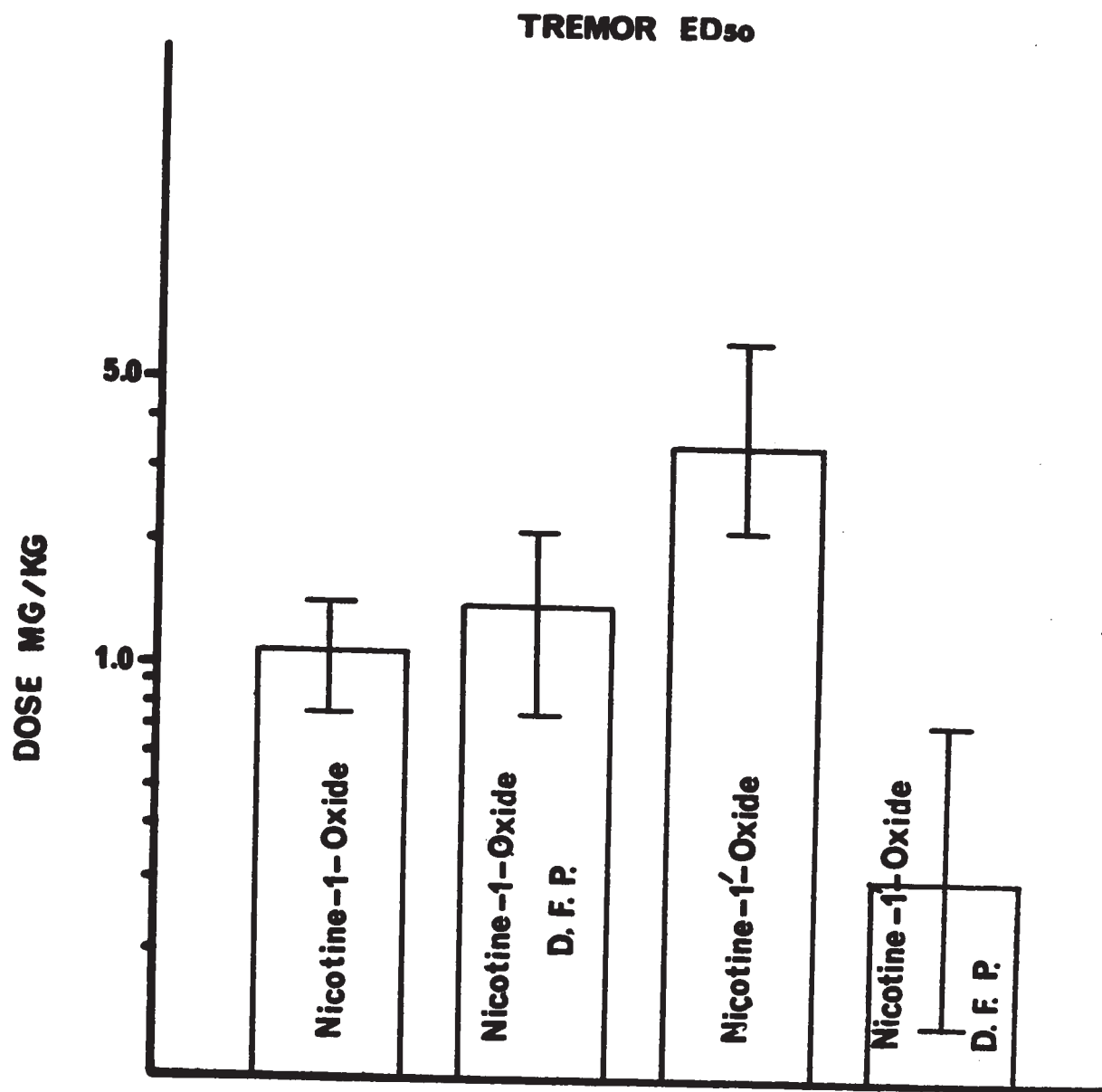
Appendix VIII

Fig. 11 The dose response lines for tremor
and or convulsions with their ED_{50} 's following
i.c. injection of nicotine-1'-oxide dihydrochloride
in normal mice and mice pretreated with $\frac{1}{2}$ LD_{50} i.v.
of D F P



Appendix VIII

Fig. III ED_{50} 's for tremor and or convulsions
with the corresponding 95% confidence limits of
i.c. injected nicotine-1-oxide sulphate and
nicotine-1'-oxide dihydrochloride in normal mice
and mice pretreated with $\frac{1}{2}$ LD_{50} of l.v. D F P



Dose response for tremor and or convulsions after i.c. nicotine-1-oxide
and nicotine-1'-oxide in mice

Drug treatment	Dose mg/kg	r	ED ₅₀ (95% confidence limits)
Nicotine-1-oxide	2.75	10/10	1.10 (0.77 - 1.46)
	1.95	8/10	
	1.08	5/10	
	0.54	1/10	
Nicotine-1-oxide 30 min after i.v. 1/2 LD ₅₀ of D F P	5.68	18/20	1.43 (0.77 - 2.18)
	2.84	14/20	
	2.30	5/10	
	0.54	5/20	
Nicotine-1'-oxide	13.4	8/10	3.58 (2.22 - 6.43)
	8.4	8/10	
	5.1	5/10	
	4.2	6/10	
	2.5	2/10	
	1.68	4/10	
	0.84	4/20	
	0.13	0/10	
Nicotine-1'-oxide 30 min after i.v. 1/2 LD ₅₀ of D F P	13.4	9/10	0.315 (0.138 - 0.752)
	4.20	9/10	
	2.52	6/10	
	1.26	5/10	
	1.04	9/10	
	0.420	4/10	
	0.126	2/10	
	0.104	6/10	
	0.042	2/10	
	0.031	3/10	
	0.0126	1/10	
	0.0104	2/10	

APPENDIX IX

Dose response for tremor and or convulsions after i.c. Nicotine -N- oxide
in mice

Drug treatment	Dose mg/kg	r	ED ₅₀ (95% confidence limits)
Nicotine -N- oxide	100.0	10/10	
	38.0	8/10	
	20.0	6/10	14.0 (8.05 - 22.2)
	10.0	5/10	
	5.0	1/10	
Nicotine -N- oxide	50.0	9/10	
30 min after atropine 15.0 mg/kg	20.0	6/10	14.6 (8.31 - 26.1)
i.p.	10.0	3/10	
	5.0	2/10	
Nicotine -N- oxide	65.0	9/10	
30 min after	55.5	5/10	
mecamylamine 1.7	28.0	3/10	37.8 (23.2 - 64.1)
mg/kg i.p.	15.0	2/10	
Nicotine -N- oxide	10.0	9/10	
30 min after 1/2	5.0	8/9	
LD ₅₀ i.v. D F P	1.0	8/10	
	0.30	4/10	0.291 (0.114 - 0.742)
	0.10	3/10	
	0.030	2/10	
	0.010	1/10	
Nicotine -N- oxide	10.0	8/10	
30 min after 1/2	5.0	5/10	3.73 (1.64 - 10.0)
LD ₅₀ i.v. D F P and	2.5	4/10	
atropine 3.0 mg/kg	1.0	2/10	
i.p.			
Nicotine -N- oxide	26.0	10/10	
30 min after 1/2	17.0	9/10	
LD ₅₀ i.v. D F P and	13.0	6/10	10.3 (8.37 - 12.4)
atropine 15.0 mg/kg	10.0	5/10	
i.p.	8.0	4/10	
	6.6	1/10	

Drug treatment	Dose mg/kg	r	ED ₅₀ (95% confidence limits)
Nicotine -N- oxide	30.0	8/9	5.22 (3.04 - 9.00)
30 min after 1/2	10.0	9/10	
LD ₅₀ i.v. D F P	5.0	4/10	
and 1 hr after i.c.	3.0	2/9	
HC-3 0.1 mg/kg.	1.0	1/10	

APPENDIX X

Dose response for tremor and or convulsions after i.c. Nicotine -N- oxide
in mice (1)

Drug treatment	Dose mg/kg	r	ED ₅₀ (95% confidence limits)
Nicotine -N- oxide	30.0	8/10	
	20.0	7/10	
	15.0	5/10	12.7 (7.7 - 20.9)
	7.5	3/10	
	3.0	1/10	
Nicotine -N- oxide	50.0	10/10	
1 hr after i.c.	20.0	7/10	10.7 (6.1 - 16.8)
HC-3 0.1 mg/kg.	10.0	5/10	
	5.0	2/10	

(1) Male mice 22-28_s from the inbred colony maintained at the Defence Research Establishment Suffield.